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Attorney Docket P1084R1C1

PATENT

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jc586 U.S. PTO

CERTIFICATION UNDER 37 CFR 1.10

EL 141 796 666 US: Express Mail Number

January 11, 2000: Date of Deposit

I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

Pamela Gavette
Pamela Gavette

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, D.C. 20231

jc678 U.S. PTO
09/480977
01/11/00

NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

Paul J. Godowski, Burlingame, California
Melanie Rose Mark Burlingame, California
Dong Xiao Zhang Burlingame, California

Title: **ErbB4 RECEPTOR-SPECIFIC NEUREGULIN RELATED LIGANDS AND USES THEREFOR**

1. Type of Application

- [] This application is for an original, non-provisional application.
- [] This is a non-provisional application claiming priority to provisional application no. __, filed __, the entire disclosure of which is hereby incorporated by reference.
- [X] This is a [] continuation-in-part [X] continuation [] divisional application claiming priority to application Serial Number 08/899,437, filed 24 July 1997, the entire disclosure of which is hereby incorporated by reference.

2. Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b) (Non-provisional)

86 pages of specification
5 pages of claims
1 page(s) of abstract
8 sheet(s) of drawings
☒ formal ☐ informal

03460977 011100

3. Declaration or Oath

(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)

☐ An executed declaration of the inventor(s) ☐ is enclosed ☐ will follow.

(for Cont./Div. where inventorship is the same or inventor(s) being deleted)

☒ A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

(for Cont./Div. where inventor(s) being deleted)

☐ A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

4. Assignment

(for new and CIP applications)

☐ An Assignment of the invention to GENENTECH, INC. ☐ is enclosed with attached Recordation Form Cover Sheet ☐ will follow.

(for cont./div.)

☒ The prior application is assigned of record to Genentech, Inc.

5. Amendments (for continuation and divisional applications)

☒ Cancel in this application original claims 2, 3, 5-38 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

☒ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119

☒ Amend the specification by inserting before the first line the sentence:

--This is a

☐ non-provisional application

☒ continuation

☐ divisional

☐ continuation-in-part

of co-pending application(s)

☒ Serial No. 08/899,437 filed on July 24, 1997, which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120. --

08/899,437 011100

_____ International Application _ filed on _ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.--

_____ provisional application No. _ filed _ , the entire disclosure of which is hereby incorporated by reference and to which application(s) priority is claimed under 35 USC §119.--

6. Fee Calculation (37 CFR 1.16)

The fee has been calculated as follows:

CLAIMS FOR FEE CALCULATION					
Number Filed		Number Extra		Rate	Basic Fee 37 CFR 1.16(a)
					\$690.00
Total Claims	2	- 20 =	0	X \$18.00	\$0.00
Independent Claims	1	- 3 =	0	X \$78.00	\$0.00
_____ Multiple dependent claim(s), if any				+ \$260.00	\$0.00
Filing Fee Calculation					\$690.00

7. Method of Payment of Fees

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of \$690.00. **A duplicate copy of this transmittal is enclosed.**

8. Authorization to Charge Additional Fees

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR §1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. **A duplicate copy of this sheet is enclosed.**

9. Additional Papers Enclosed

- ☐ Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
- ☐ Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
- ☐ A new Power of Attorney or authorization of agent.
- ☐ Other:

10. Maintenance of Copendency of Prior Application (for continuation and divisional applications)

[This item must be completed and the necessary papers filed in the prior application if the period set in the prior application has run]

_____ A petition, fee and/or response has been filed to extend the term in the pending prior application until

0043067 01100

_____ A copy of the petition for extension of time in the **prior** application is attached.

11. Correspondence Address:

X Address all future communications to:

GENENTECH, INC.
Attn: Deirdre L. Conley, Ph.D.
1 DNA Way
South San Francisco, CA 94080-4990
(650) 225-2066


Respectfully submitted,
GENENTECH, INC.

Date: January 10, 2000

By: Deirdre L. Conley
Deirdre L. Conley, Ph.D.
Reg. No. 36,487

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Fax: (650) 952-9881

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<p>In re Application of</p> <p>Paul J. Godowski et al.</p> <p>Serial No.: Unassigned</p>	<p>Group Art Unit: Unassigned</p> <p>Examiner: Unassigned</p>
<p>Filed: 11 January 2000</p> <p>For: ErbB4 RECEPTOR-SPECIFIC NEUREGULIN RELATED LIGANDS AND USES THEREFOR</p>	<p>CERTIFICATION UNDER 37 CFR 1.10</p> <p><u>EL 141 796 666 US</u>: Express Mail Number <u>11 January 2000</u>: Date of Deposit.</p> <p>I hereby certify that this correspondence, consisting of attached papers, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.</p> <p> Pamela Gavette</p>

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to review of the above-referenced application on the merits, please amend the application as follows:

IN THE SPECIFICATION

Please amend the specification as follows:

On page 1, line 10, after "under" insert - 35 USC 120 to non-provisional application Serial No. 08/899,437 filed July 24, 1997, which claims priority-.

On page 1, line 10, delete "60/ _____ (to be assigned)" and insert therefor -60/052,019-.

On page 1, line 11, after "contents of which" insert -applications-.

On page 12, line 14, delete "Aeischer" and insert therefor -Aebischer-.

On page 12, line 14, after "Nature" insert -Medicine-.

On page 85, beginning on line 24, delete "12301 Parklawn Drive, Rockville, MD, USA" and insert therefor -10801 University Boulevard, Manassas, VA 20110-2209-.

On page 10, line 4, delete "ATCC _____" and insert --ATCC 209156--.

On page 10, line 7, delete "ATCC _____" and insert --ATCC 209157--.

On page 10, lines 9 and 10, delete "ATCC _____" and insert --ATCC 209297--.

On page 12, line 14, delete "Aischer" and insert therefor --Aebischer--.

On page 12, line 14, after "Nature" insert --Medicine--.

On page 30, line 20, delete "ATCC _____" and insert --ATCC 209156--.

On page 30, lines 20 and 21, delete "ATCC _____" and insert --ATCC 209157--.

On page 30, line 21, delete "pRK5.tk.neo.hNRG3" and insert --pRK5.tk.neo.hNRG3B2--.

On page 30, line 21, delete "ATCC _____" and insert --ATCC 209297--.

On page 72, lines 26 and 27, delete "ATCC _____" and insert --ATCC 209157--.

On page 73, line 15, delete "ATCC _____" and insert --ATCC 209156--.

On page 85, line 26, insert --209156-- as the ATCC Deposit No. and --July 22, 1997-- as the Deposit Date.

On page 85, line 28, insert --209157-- as the ATCC Deposit No. and --July 22, 1997-- as the Deposit Date.

On page 85, line 30, insert --209297-- as the ATCC Deposit No. and --September 23, 1997-- as the Deposit Date.

On page 88, claim 6, second line, delete "ATCC deposit _____" and insert --ATCC deposit 209156--.

On page 88, claim 7, second line, delete "ATCC deposit _____" and insert --ATCC deposit 209157--.

On page 88, claim 8, second line, delete "ATCC deposit _____" and insert --ATCC deposit 209297--.

On page 89, claim 19, delete "ATCC _____" and insert --ATCC 209156--.

On page 89, claim 19, delete "pLXSN.mNRG" and insert --pLXSN.mNRG3--.

On page 89, claim 20, delete "ATCC _____" and insert --ATCC 209157--.

On page 89, claim 21, delete "ATCC _____" and insert --ATCC 209297--.

Please further amend the specification by renumbering pages 87-92 to be pages 112-117. Please further amend the specification by inserting after page 86 the attached Sequence Listing as pages 87-111.

SEQUENCE LISTING

Pursuant to 37 CFR § 1.821(f) and (g), the undersigned hereby states that the paper copy and computer readable form are the same and that the Sequence Listing submitted herewith includes no

new matter.

IN THE CLAIMS

The claims are reiterated for the convenience of the Examiner. Please amend the claims as follows:

1. (Amended) [A] An isolated polypeptide comprising an amino acid sequence encoding [an] the EGF-like domain of SEQ ID NO:4[, wherein the amino acid sequence has the binding characteristics of NRG3].

Cancel claims 2 and 3 without prejudice to later prosecution.

4. (Amended) The polypeptide of claim 1, wherein the polypeptide binds to the ErbB4 receptor and [stimulates] activates tyrosine phosphorylation of the ErbB4 receptor.

Cancel claims 5-38 without prejudice to later prosecution.

REMARKS

Formal Matters

The specification is amended to claim priority to previous applications in this series. Support for the amendment is found in the attached copy of the corrected filing receipt for application Serial No. 08/899,437. Further amendment to the specification corrects a typographical error of an author's name and a journal name, finding support in the June 9, 1998 IDS, item 6, and the paper copy of the reference submitted in the parent application. Additional amendment to the specification corrects the address of the ATCC. No new matter is added by the amendments to the specification.

Claims 1 and 4 are pending in the application. Claims 2, 3, and 5-38 are canceled without prejudice to later prosecution. Prosecution and allowance of claims 1 and 4 is pursued following allowance of claims 5-11 in the parent application Serial No. 08/899,437.

Amendments to the claims are made to recite an isolated polypeptide, amino acid sequences, activation of receptor tyrosine phosphorylation. The amendments are made merely to expedite prosecution by incorporating amendments made in the parent application. Applicants' traversal of rejections in the parent application are of record in that case and are herein incorporated by reference. As indicated in the parent case, support for the amendments is found throughout the specification,

such as at page 78, lines 16-19; page 80, line 26 to page 81, line 22; SEQ ID NO:4; and the attached ATCC deposit receipts. No new matter is added by the amendments to the claims.

CONCLUSION

Applicants respectfully submit that the claims are in condition for allowance. It is therefore earnestly solicited that such a final favorable disposition is made. The Examiner is invited to telephone Deirdre L. Conley, Ph.D., (Reg. No. 36,487) at (650) 225-2066 if deemed helpful to clarify and advance prosecution.

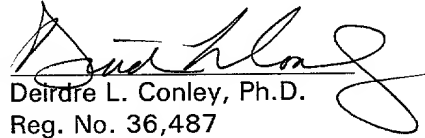
This Preliminary Amendment is submitted with a transmittal letter. In the unlikely event that this document is separated from the transmittal letter, Applicants hereby petition the Commissioner to authorize charging our Deposit Account 07-0630 for an fees required or credits due and any extensions of time necessary to maintain the pendency of this application.

Respectfully submitted,

GENENTECH, INC.

Date: January 10, 2000

By:


Deirdre L. Conley, Ph.D.
Reg. No. 36,487

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Attorney Docket P1084R1
Express Mail No. EL 141 796 666 US
Mailed: 11 January 2000

Patent Docket No. P1084R1
Express Mail No. EM239527155US
Mailed: July 24, 1997

5 **ErbB4 RECEPTOR-SPECIFIC NEUREGULIN RELATED LIGANDS
AND USES THEREFOR**

RELATED APPLICATION

10 This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming
priority under 35 USC 119(e) to provisional application number 60_____ (to be assigned)
filed July 9, 1997, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

 The present invention concerns novel neuregulin related ligands. More particularly, the
invention relates to a new member of the neuregulin family and functional derivatives of the
novel polypeptide.

BACKGROUND OF THE INVENTION

20 Signal transduction affecting cell growth and differentiation is regulated in part by
phosphorylation of various cellular proteins. Protein tyrosine kinases are enzymes that catalyze
this process. Receptor protein tyrosine kinases are believed to direct cellular growth via ligand-
stimulated tyrosine phosphorylation of intracellular substrates. Growth factor receptor protein
tyrosine kinases of the class I subfamily include the 170 kDa epidermal growth factor receptor
(EGFR) encoded by the *erbB1* gene. *erbB1* has been causally implicated in human malignancy.
25 In particular, increased expression of this gene has been observed in more aggressive carcinomas
of the breast, bladder, lung and stomach. The second member of the class I subfamily, p185^{neu},
was originally identified as the product of the transforming gene from neuroblastomas of
chemically treated rats. The *neu* gene (also called *erbB2* and HER2) encodes a 185 kDa receptor
protein tyrosine kinase. Amplification and/or overexpression of the human HER2 gene
30 correlates with a poor prognosis in breast and ovarian cancers (Slamon *et al.*, (1987) Science

235:177-182; and Slamon *et al.*, (1989) Science 244:707-712). Overexpression of HER2 has been correlated with other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon and bladder. A further related gene, called *erbB3* or HER3, has also been described (Kraus *et al.*, (1989) Proc. Natl. Acad. Sci. USA 86:9193-9197). Kraus *et al.* (1989) discovered that markedly elevated levels of *erbB3* mRNA were present in certain human mammary tumor cell lines indicating that *erbB3*, like *erbB1* and *erbB2*, may play a role in human malignancies. The *erbB3* gene has been found to be overexpressed in breast (Lemoine *et al.* (1992) Br. J. Cancer 66:1116-1121), gastrointestinal (Poller *et al.* (1992) J. Pathol. 168:275-280, Rajkumar *et al.* (1993) J. Pathol. 170:271-278, and Sanidas *et al.* (1993) Int. J. Cancer 54:935-940, and pancreatic cancers (Lemoine *et al.* (1992) J. Pathol. 168:269-273, and Friess *et al.* (1995) Clinical Cancer Research 1:1413-1420).

The class I subfamily of growth factor receptor protein tyrosine kinases has been further extended to include the HER4/Erb4 receptor (EP Pat Appln No 599,274; Plowman *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:1746-1750; and Plowman *et al.* (1993) Nature 366:473-475. Plowman *et al.* found that increased HER4 expression closely correlated with certain carcinomas of epithelial origin, including breast adenocarcinomas. Diagnostic methods for detection of human neoplastic conditions (especially breast cancers) which evaluate HER4 expression are described in EP Pat Appln No. 599,274.

The quest for the activator of the HER2 oncogene has lead to the discovery of a family of polypeptides, collectively called neuregulins (NRG1). These proteins appear to result from alternate splicing of a single gene which was mapped to the short arm of human chromosome 8 by Orr-Urtreger *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:1867-1871.

Holmes *et al.* isolated and cloned a family of polypeptide activators for the HER2 receptor which they called heregulin- α (HRG- α), heregulin- β 1 (HRG- β 1), heregulin- β 2 (HRG- β 2), heregulin- β 2-like (HRG- β 2-like), and heregulin- β 3 (HRG- β 3). See Holmes *et al.* (1992) Science 256:1205-1210; WO 92/20798; and U.S. Patent 5,367,060. The 45 kDa polypeptide, HRG- α , was purified from the conditioned medium of the MDA-MB-231 human breast cancer cell line. These researchers demonstrated the ability of the purified heregulin polypeptides to activate tyrosine phosphorylation of the HER2 receptor in MCF7 breast tumor cells.

Furthermore, the mitogenic activity of the heregulin polypeptides on SK-BR-3 cells (which express high levels of the HER2 receptor) was illustrated. Like other growth factors which belong to the EGF family, soluble HRG polypeptides appear to be derived from a membrane bound precursor (called pro-HRG) which is proteolytically processed to release the 45 kDa soluble form. These pro-HRGs lack a N-terminal signal peptide.

While heregulins are substantially identical in the first 213 amino acid residues, they are classified into two major types, α and β , based on two variant EGF-like domains which differ in their C-terminal portions. Nevertheless, these EGF-like domains are identical in the spacing of six cysteine residues contained therein. Based on an amino acid sequence comparison, Holmes *et al.* found that between the first and sixth cysteines in the EGF-like domain, HRGs were 45% similar to heparin-binding EGF-like growth factor (HB-EGF), 35% identical to amphiregulin (AR), 32% identical to TGF- α , and 27% identical to EGF.

The 44 kDa *neu* differentiation factor (NDF), which is the rat equivalent of human HRG, was first described by Peles *et al.* (1992) Cell 69:205-216; and Wen *et al.* (1992) Cell 69:559-572. Like the HRG polypeptides, NDF has an immunoglobulin (Ig) homology domain followed by an EGF-like domain and lacks a N-terminal signal peptide. Subsequently, Wen *et al.* (1994) Mol. Cell. Biol. 14(3):1909-1919 carried out cloning experiments to extend the family of NDFs. This work revealed six distinct fibroblastic pro-NDFs. Adopting the nomenclature of Holmes *et al.*, the NDFs are classified as either α or β polypeptides based on the sequences of the EGF-like domains. Isoforms 1 to 4 are characterized on the basis of the region between the EGF-like domain and transmembrane domain. Also, isoforms a, b and c are described which have variable length cytoplasmic domains. These researchers conclude that different NDF isoforms are generated by alternative splicing and perform distinct tissue-specific functions. See also EP 505 148; WO 93/22424; and WO 94/28133 concerning NDF.

Falls *et al.* (1993) Cell 72:801-815 describe another member of the heregulin family which they call acetylcholine receptor inducing activity (ARIA) polypeptide. The chicken-derived ARIA polypeptide stimulates synthesis of muscle acetylcholine receptors. See also WO 94/08007. ARIA is a β -type heregulin and lacks the entire spacer region rich in glycosylation sites between the Ig-like domain and EGF-like domain of HRG α , and HRG β 1- β 3.

Marchionni *et al.* identified several bovine-derived proteins which they call glial growth factors (GGFs) (Marchionni *et al.* (1993) *Nature* 362:312-318). These GGFs share the Ig-like domain and EGF-like domain with the other heregulin proteins described above, but also have an amino-terminal kringle domain. GGFs generally do not have the complete glycosylated spacer region between the Ig-like domain and EGF-like domain. Only one of the GGFs, GGFII, possessed a N-terminal signal peptide. See also WO 92/18627; WO 94/00140; WO 94/04560; WO 94/26298; and WO 95/32724 which refer to GGFs and uses thereof.

Ho *et al.* in (1995) *J. Biol. Chem.* 270(4):14523-14532 describe another member of the heregulin family called sensory and motor neuron-derived factor (SMDF). This protein has an EGF-like domain characteristic of all other heregulin polypeptides but a distinct N-terminal domain. The major structural difference between SMDF and the other heregulin polypeptides is the lack in SMDF of the Ig-like domain and the "glyco" spacer characteristic of all the other heregulin polypeptides. Another feature of SMDF is the presence of two stretches of hydrophobic amino acids near the N-terminus.

While the heregulin polypeptides were first identified based on their ability to activate the HER2 receptor (see Holmes *et al.*, *supra*), it was discovered that certain ovarian cells expressing *neu* and *neu*-transfected fibroblasts did not bind or crosslink to NDF, nor did they respond to NDF to undergo tyrosine phosphorylation (Peles *et al.* (1993) *EMBO J.* 12:961-971). This indicated that another cellular component was necessary for conferring full heregulin responsiveness. Carraway *et al.* subsequently demonstrated that ^{125}I -rHRG β 1₁₇₇₋₂₄₄ bound to NIH-3T3 fibroblasts stably transfected with bovine *erbB3* but not to non-transfected parental cells. Accordingly, they conclude that ErbB3 is a receptor for HRG and mediates phosphorylation of intrinsic tyrosine residues as well as phosphorylation of ErbB2 receptor in cells which express both receptors. Caraway *et al.* (1994) *J. Biol. Chem.* 269(19):14303-14306. Sliwkowski *et al.* (1994) *J. Biol. Chem.* 269(20):14661-14665 found that cells transfected with HER3 alone show low affinities for heregulin, whereas cells transfected with both HER2 and HER3 show higher affinities.

This observation correlates with the "receptor cross-talking" described previously by Kokai *et al.*, *Cell* 58:287-292 (1989); Stern *et al.* (1988) *EMBO J.* 7:995-1001; and King *et al.*,

4:13-18 (1989). These researchers found that binding of EGF to the EGFR resulted in activation of the EGFR kinase domain and cross-phosphorylation of p185^{HER2}. This is believed to be a result of ligand-induced receptor heterodimerization and the concomitant cross-phosphorylation of the receptors within the heterodimer (Wada *et al.* (1990) *Cell* 61:1339-1347).

5 Plowman and his colleagues have similarly studied p185^{HER4}/p185^{HER2} activation. They expressed p185^{HER2} alone, p185^{HER4} alone, or the two receptors together in human T lymphocytes and demonstrated that heregulin is capable of stimulating tyrosine phosphorylation of p185^{HER4}, but could only stimulate p185^{HER2} phosphorylation in cells expressing both receptors. Plowman *et al.*, *Nature* 336:473-475 (1993). Thus, heregulin is the only known example of a member of the EGF growth factor family that can interact with several receptors. Carraway and Cantley
10 (1994) *Cell* 78:5-8.

The biological role of heregulin has been investigated by several groups. For example, Falls *et al.*, (discussed above) found that ARIA plays a role in myotube differentiation, namely affecting the synthesis and concentration of neurotransmitter receptors in the postsynaptic muscle cells of motor neurons. Corfas and Fischbach demonstrated that ARIA also increases the number of sodium channels in chick muscle. Corfas and Fischbach (1993) *J. Neuroscience* 13(5): 2118-2125. It has also been shown that GGFII is mitogenic for subconfluent quiescent human myoblasts and that differentiation of clonal human myoblasts in the continuous presence of GGFII results in greater numbers of myotubes after six days of differentiation (Sklar *et al.* (1994) *J. Cell Biochem.*, Abst. W462, 18D, 540). See also WO 94/26298 published November 24,
20 1994.

Holmes *et al.*, *supra*, found that HRG exerted a mitogenic effect on mammary cell lines (such as SK-BR-3 and MCF-7). The mitogenic activity of GGFs on Schwann cells has also been reported. See, e.g., Brockes *et al.* (1980) *J. Biol. Chem.* 255(18):8374-8377; Lemke and Brockes (1984) *J. Neurosci.* 4:75-83; Brockes *et al.* (1984) *J. Neuroscience* 4(1):75-83; Brockes *et al.* (1986) *Ann. Neurol.* 20(3):317-322; Brockes, J. (1987) *Methods in Enzym.* 147:217-225 and Marchionni *et al.*, *supra*. Schwann cells constitute important glial cells which provide myelin sheathing around the axons of neurons, thereby forming individual nerve fibers. Thus, it is
25 apparent that Schwann cells play an important role in the development, function and regeneration

of peripheral nerves. The implications of this from a therapeutic standpoint have been addressed by Levi *et al.* (1994) *J. Neuroscience* 14(3):1309-1319. Levi *et al.* discuss the potential for construction of a cellular prosthesis comprising human Schwann cells which could be transplanted into areas of damaged spinal cord. Methods for culturing Schwann cells *ex vivo* have been described. See WO 94/00140 and Li *et al.* (1996) *J. Neuroscience* 16(6):2012-2019.

Pinkas-Kramarski *et al.* found that NDF seems to be expressed in neurons and glial cells in embryonic and adult rat brain and primary cultures of rat brain cells, and suggested that it may act as a survival and maturation factor for astrocytes (Pinkas-Kramarski *et al.* (1994) *PNAS*, USA 91:9387-9391). Meyer and Birchmeier (1994) *PNAS*, USA 91:1064-1068 analyzed expression of heregulin during mouse embryogenesis and in the perinatal animal using *in situ* hybridization and RNase protection experiments. These authors conclude that, based on expression of this molecule, heregulin plays a role *in vivo* as a mesenchymal and neuronal factor. Also, their findings imply that heregulin functions in the development of epithelia. Similarly, Danilenko *et al.* (1994) Abstract 3101, *FASEB* 8(4-5):A535, found that the interaction of NDF and the HER2 receptor is important in directing epidermal migration and differentiation during wound repair.

Although NRG1 was initially proposed to be the ligand for the receptor tyrosine kinase ErbB2, further studies have demonstrated that activation of ErbB2 frequently occurred as a result of NRG1 binding to ErbB3 (Sliwkowski, M.X., et al. (1994) *J. Biol. Chem.* 269:14661-14665) or ErbB4 (Plowman, G.D. et al. (1993) *Nature* 366:473-475; and Carraway, K.L. and Cantley, L.C. (1994) *Cell* 78:5-8) receptors. Recent studies have begun to highlight the roles of NRG1, ErbB2 receptor and ErbB4 receptor in the development of the heart. Mice lacking ErbB4 receptor, ErbB2 receptor or NRG1 die during mid-embryogenesis (embryonic day 10.5) from the aborted development of myocardial trabeculae in the ventricle (Meyer & Birchmeier (1995) *Nature* 378:386-90; Gassmann *et al.* (1995) *Nature* 378:390-4; and Lee *et al.* (1995) *Nature* 378:394-8). These results are consistent with the view that NRG1, expressed in the endocardium, is an important ligand required for the activation of ErbB2 and ErbB4 receptors in the myocardium.

These same studies suggest that NRG1 and ErbB2 receptor may play a different role than ErbB4 receptor in the development of the hind brain. NRG1 is expressed in the neuroepithelium

and cells arising from rhombomeres 2, 4 and 6, while ErbB4 receptor is expressed in rhombomeres 3 and 5. NRG1 and ErbB2 receptor knockout mice exhibit a loss of cells and axons of the cranial sensory ganglia. In contrast, ErbB4 receptor deficient mice do not exhibit a loss of cellularity in the cranial ganglia. Rather, the organization, spacing and pattern of innervation of these ganglia to and from the central nervous system is disrupted (Gassmann *et al.*, *supra*). One possible reason for this difference in hindbrain phenotypes of NRG1 and ErbB4 receptor knockout mice is that additional ligand(s) distinct from NRG1 may be recognized by ErbB4 in the CNS (Gassmann *et al.*, *supra*).

SUMMARY OF THE INVENTION

The present invention is based on the identification, recombinant production and characterization of a novel member of the family of neuregulins (NRG1). More specifically, the invention concerns a novel polypeptide, NRG3, comprising an EGF-like domain distinct from EGF-like domains of NRG1 and NRG2. In addition, the NRG3 disclosed herein displays distinct receptor binding characteristics relative to other neuregulin-like polypeptides.

In analyzing the homologous sequence motif, homology to the EGF-like domain of NRG1 was observed in the subset of amino acids that are conserved in most neuregulins. Based upon this observation and the observed ErbB4 receptor binding characteristics, the novel protein, NRG3, has been identified as a new member of the family of neuregulins. The novel protein contains domains that are distantly related to, but distinct from, those found in the other members of the NRG1 family. In addition, it is expressed primarily in embryonic and adult tissues of the central nervous system. NRG3 represents a novel member of the neuregulin family of compounds, members of which are involved in cell proliferation and differentiation, epithelial development, cardiac development, neurological development, as well as acting as glial cell mitogens, and as mesenchymal and neuronal factors.

In one aspect, the present invention concerns a novel isolated mammalian NRG3 polypeptide having an EGF-like domain, and functional derivatives of the novel NRG3, which polypeptides bind the ErbB4 receptor. The native polypeptides within the scope of the present invention are characterized as containing an extracellular domain including an EGF-like domain,

a transmembrane domain and a cytoplasmic domain. The present invention specifically includes the soluble forms of the novel NRG3 ligand molecules of the invention, which have a transmembrane domain that cannot associate with a cell membrane, and optionally devoid of all or part of the cytoplasmic domain. By "transmembrane domain" is meant a domain of the polypeptide that contains a sufficient number of hydrophobic amino acids to allow the polypeptide to insert and anchor in a cell membrane. By "transmembrane domain that cannot associate with a cell membrane" is meant a transmembrane domain that has been altered by mutation or deletion such that is insufficiently hydrophobic to allow insertion or other association with a cell membrane. Such a transmembrane domain does not preclude, for example, the fusion of the NRG3 of the invention, or fragment thereof, with a secretion signal sequence useful for secretion of the polypeptide from the cell, an insufficient number of hydrophobic amino acid side chains are present devoid of an active transmembrane domain does not insert into a cell membrane. Mutations or alterations of the amino acid sequence useful to achieve an inactive transmembrane domain include, but are not limited to, deletion or substitution of amino acids within the transmembrane domain.

In a particular embodiment, the invention concerns isolated polypeptides, preferably NRG3 ligands, having at least 75% amino acid identity to polypeptides selected from the group consisting of

- (1) a polypeptide comprising the amino acid sequence encoding the EGF-like domain shown in Figure 3 (SEQ ID NO:4);
- (2) a polypeptide comprising the amino acid sequence encoding the extracellular domain of mouse or human NRG3 shown in Figure 3 (SEQ ID NO: 3 or SEQ ID NO:7, respectively);
- (3) a polypeptide comprising the amino acid sequence of the native mouse or human NRG3 polypeptide shown in Figure 3 (SEQ ID NO: 2 and SEQ ID NO:6, respectfully);
- (4) a further mammalian homologue of polypeptide (1) - (3);
- (5) a soluble form of any of the polypeptides (1) - (4) devoid of an active transmembrane domain; and

(6) a derivative of any of the polypeptides (1) - (5), retaining the qualitative EGF-like domain and NRG3 receptor binding properties of a polypeptide (1) - (5).

While the native NRG3 polypeptides of the present invention are glycoproteins, the present invention also encompasses variant molecules unaccompanied by native glycosylation or having a variant glycosylation pattern. Preferably, the EGF-like domain of the NRG3 polypeptide is unglycosylated.

In a further embodiment, the invention includes an antagonist of a novel NRG3 of the present invention. The antagonist of the invention may be a peptide that binds an NRG3 such as an anti-NRG3 antibody or binding fragment thereof. Preferably, the NRG3 antagonist of the invention substantially reduces binding of a natural ErbB4 receptor ligand, such as an NRG3, to the ErbB4 receptor, thereby preventing or limiting activation of the receptor. In a preferred embodiment, the antagonist reduces NRG3 binding to its receptor to less than 50%, preferably less than 20%, most preferably less than 10% of the binding of an NRG3 under like conditions.

In yet another embodiment, the invention includes an agonist of a novel NRG3 of the present invention. The agonist of the invention may be a NRG3, or it may be an anti-NRG3 receptor antibody or receptor binding fragment. An agonist NRG3 of the invention may also be a polypeptide encoded by an alternatively spliced form of the native NRG3-encoding gene, preferably comprising the NRG3 EGF-like domain disclosed herein. In an embodiment of the agonist of the invention, the NRG3 agonist is an anti-ErbB4 receptor antibody, which antibody binds to and activates the ErbB4 receptor. Preferably, the binding affinity of the agonist is at least 25% of the affinity of the native ligand, more preferably at least 50%, and most preferably at least 90% of the affinity of the native ligand. Similarly, it is preferred that the agonist of the invention activate the ErbB4 receptor at the level of at least 25%, more preferably at least 50%, most preferably at least 90% of activation of the native NRG3.

The invention further concerns a nucleic acid molecule encoding a novel NRG3 of the present invention, vectors containing such nucleic acid, and host cells transformed with the vectors. The nucleic acid preferably encodes at least the EGF-like domain of a native or variant ErbB4 receptor-specific NRG3 of the present invention. The invention further includes nucleic acids hybridizing under stringent conditions to the complement of a nucleic acid encoding a

native ErbB4 receptor-specific NRG3 of the present invention, and encoding a protein retaining the qualitative ErbB4 receptor-specific binding properties of a native NRG3 disclosed herein. In addition, the invention includes a nucleic acid deposited with the American Type Culture Collection as ATCC _____ (pLXSN.mNRG3), which nucleic acid is an expression vector comprising nucleic acid encoding the mouse NRG3 open reading frame (SEQ ID NO:1). The invention also includes a nucleic acid deposited with the American Type Culture Collection as ATCC _____ (pRK5.tk.neo.hNRG3B1), which nucleic acid is an expression vector comprising nucleic acid encoding a human NRG3 nucleic acid (SEQ ID NO:5). The invention also includes a nucleic acid deposited with the American Type Culture Collection as ATCC _____ (pRK5.tk.neo.hNRG3B2), which nucleic acid is an expression vector comprising nucleic acid encoding an alternatively spliced form of human NRG3 nucleic acid (SEQ ID NO:22) lacking nucleic acids 1585 to 1656 of SEQ ID NO:5. The deduced amino acid sequence of the alternatively spliced human NRG3B2 is found in SEQ ID NO:23 which lacks amino acids 529 to 552 of SEQ ID NO:6. A comparison of the hNRG3B1 and hNRG3B2 amino acid sequences is shown in Fig. 4B. The invention further includes NRG3 amino acid sequences of mouse and human NRG3, alternatively spliced forms or fragments thereof, encoded by the deposited expression vectors.

In another aspect, the invention concerns a process for producing a NRG3 of the invention, which process comprises transforming a host cell with nucleic acid encoding the desired NRG3, culturing the transformed host cell and recovering the NRG3 produced from the host cell or host cell culture.

As an alternative to production of the NRG3 in a transformed host cell, the invention provides a method for producing NRG3 comprising: (a) transforming a cell containing an endogenous NRG3 gene with a homologous DNA comprising an amplifiable gene and a flanking sequence of at least about 150 base pairs that is homologous with a DNA sequence within or in proximity to the endogenous NRG3 gene, whereby the homologous DNA integrates into the cell genome by recombination; (b) culturing the cell under conditions that select for amplification of the amplifiable gene, whereby the NRG3 gene is also amplified; and thereafter (c) recovering NRG3 from the cell.

In a further aspect, the invention concerns an antibody that binds specifically to a NRG3 of the present invention, and to a hybridoma cell line producing such an antibody.

In a still further aspect, the invention concerns an immunoadhesin comprising a novel NRG3 sequence, as disclosed herein, fused to an immunoglobulin sequence. The NRG3 sequence is preferably a transmembrane-domain-deleted form of a native or variant polypeptide fused to an immunoglobulin constant domain sequence, and comprises at least the EGF-like domain of the extracellular domain of a native NRG3 of the present invention. In another preferred embodiment, the NRG3 sequence present in the immunoadhesin shows at least about 80% sequence homology with the extracellular domain of the sequence shown in SEQ ID NO:3 NRG3 or SEQ ID NO:7 for mouse or human NRG3, respectively. The immunoglobulin constant domain sequence preferably is that of an IgG-1, IgG-2 or IgG-3 molecule, but may also be an IgA or IgM molecule.

In a further aspect, the invention encompasses a transgenic animal comprising an altered NRG3 gene in which the polypeptide encoded by the altered gene is not biologically active (non-functional), deleted, or has no more than 70% wild type activity, preferably no more than 50% activity and more preferably no more than 25% activity of the native NRG3 polypeptide. In addition, a transgenic animal of the invention includes a transgenic animal comprising and expressing a native NRG3, alternatively spliced form of NRG3, or a fragment or variant thereof. Such transgenic animals are useful for the screening of potential NRG3 agonists and antagonists.

The invention further concerns pharmaceutical compositions comprising a NRG3 as hereinabove defined in admixture with a pharmaceutically acceptable carrier. Dosages and administration of NRG3 in a pharmaceutical composition may be determined by one of ordinary skill in the art of clinical pharmacology or pharmacokinetics (see, for example, Mordenti, J. and Rescigno, A. (1992) *Pharmaceutical Research* 9:17-25; Morenti, J. *et al.* (1991) *Pharmaceutical Research* 8:1351-1359; and Mordenti, J. and Chappell, W. (1989) "The use of interspecies scaling in toxicokinetics" in Toxicokinetics and New Drug Development, Yacobi *et al.* (eds), Pergamon Press, NY, pp. 42-96, each of which references are herein incorporated by reference in its entirety).

In an aspect of the invention, the isolated nucleic acid encoding the NRG3 of the

invention, or fragment thereof, may also be used for *in vivo* or *ex vivo* gene therapy.

In an embodiment of the invention, a nucleic acid sequence encoding an NRG3, or fragment or variant thereof, is introduced into a cell of an animal as part of an expression cassette such that the NRG3-encoding nucleic acid sequence is expressed in the cell. Preferably, the NRG3 encoding nucleic acid sequence comprises sequences (such as a promotor sequence) for the control of NRG3 expression within the cell. Preferably, the expression cassette comprises a retroviral vector for delivery of the nucleic acid sequence to a cell of the animal.

In a further embodiment of the invention, a host cell expressing an NRG3 or NRG3 agonist is introduced into an animal, preferably a human, such that NRG3 or NRG3 agonist produced by the host cell is effective in treating a disorder responsive to increased local or systemic NRG3 administration. Cells genetically engineered to express an NRG3, fragment or variant thereof, can be implanted in the host to provide effective levels of factor or factors. The cells can be prepared, encapsulated, and implanted as provided in U.S. Patents 4,892,538, and 5,011,472, WO 92/19195, WO 95/05452, or Aeischer *et al.* (1996) *Nature* 2:696-699, for example, which references are herein incorporated by reference in their entirety.

The present invention includes methods of enhancing survival, proliferation or differentiation of cells comprising the ErbB4 receptor *in vivo* and *in vitro*. Normally, the cells will be treated with the NRG3 polypeptide or fragment or variant thereof. However, gene therapy approaches have been described in the art and are encompassed by the present invention. These techniques include gene delivery to a cell using adenovirus, herpes simplex I virus or adeno-associated virus as well as lipid-based delivery systems (*e.g.* liposomes). Retroviruses are useful for *ex vivo* gene therapy approaches. Accordingly, it is possible to administer the nucleic acid encoding NRG3, resulting in expression of the NRG3 polypeptide, fragment or variant in the patient or in tissue culture. For exemplary gene therapy techniques see WO 93/25673 and the references cited therein.

An aspect of the invention is a method of treating a disorder by administering to a mammal a cell encoding an NRG3 or fragment thereof, or agonist or antagonist of the NRG3 as necessary to treat the disorder, which cell secretes the NRG3 of the invention. An embodiment of the invention is a method for preventing or treating damage to a nerve or damage

to other NRG3-expressing or NRG3-responsive cells, e.g. brain, heart, or kidney cells, which method comprises implanting cells that secrete NRG3, or fragment or agonist thereof, or antagonist as may be required for the particular condition, into the body of patients in need thereof.

5 A further embodiment of the invention includes an implantation device, for preventing or treating nerve damage or damage to other cells as taught herein, containing a semipermeable membrane and a cell that secretes NRG3, or fragment or agonist thereof, (or antagonist as may be required for the particular condition) encapsulated within the membrane, the membrane being permeable to NRG3, or fragment agonist thereof, and impermeable to factors from the patient detrimental to the cells. The patient's own cells, transformed to produce NRG3 *ex vivo*, could be
10 implanted directly into the patient, optionally without such encapsulation. The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished readily as is known in the art.

15 In accordance with the *in vitro* methods of the invention, cells comprising the ErbB4 receptor are placed in a cell culture medium. Examples of ErbB4-receptor-containing cells include neural cells, *e.g.*, brain cells (such as neurons of the neocortex, cerebellum and hippocampus); cardiac cells; skeletal and smooth muscle cells; and cultured cells transformed with a recombinant NRG3.

20 Suitable tissue culture media are well known to persons skilled in the art and include, but are not limited to, Minimal Essential Medium (MEM), RPMI-1640, and Dulbecco's Modified Eagle's Medium (DMEM). These tissue culture medias are commercially available from Sigma Chemical Company (St. Louis, MO) and GIBCO (Grand Island, NY). The cells are then cultured in the cell culture medium under conditions sufficient for the cells to remain viable and
25 grow in the presence of an effective amount of NRG3. The cells can be cultured in a variety of ways, including culturing in a clot, agar, or liquid culture.

The cells are cultured at a physiologically acceptable temperature such as 37° C, for example, in the presence of an effective amount of NRG3, fragment or variant. The amount of NRG3 may vary, but preferably is in the range of about 0.1 ng/ml to about 1 mg/ml preferably

about 0.1 ng/ml to about 0.1 ng/ml. The NRG3 can of course be added to the culture at a dose determined empirically by those in the art without undue experimentation. The concentration of NRG3 in the culture will depend on various factors, such as the conditions under which the cells and NRG3 are cultured. The specific temperature and duration of incubation, as well as other culture conditions, can be varied depending on such factors as, *e.g.*, the concentration of the NRG3, and the type of cells and medium. Those skilled in the art will be able to determine operative and optimal culture conditions without undue experimentation. Proliferation, differentiation and/or survival of the cells (*e.g.* neurons) in the cultures can be determined by various assays known in the art such as those described above.

It is contemplated that using NRG3 to enhance cell survival, growth and/or differentiation *in vitro* will be useful in a variety of ways. For instance, neural cells cultured *in vitro* in the presence of NRG3 can be infused into a mammal suffering from reduced levels of the cells. Stable *in vitro* cultures can also be used for isolating cell-specific factors and for expression of endogenous or recombinantly introduced proteins in the cell. NRG3, fragments or variants thereof may also be used to enhance cell survival, proliferation and/or differentiation of cells which support the growth and/or differentiation of other cells in cell culture.

The invention also provides *in vivo* uses for NRG3. Based on the neuronal cell expression pattern of NRG3, it is believed that this molecule will be particularly useful for treating diseases which involve neural cell growth such as demyelination, or damage or loss of glial cells (*e.g.* multiple sclerosis).

The invention further provides a method for treating a mammal comprising administering a therapeutically effective amount of NRG3, NRG3 fragment, or NRG3 agonist to the mammal. For example, the mammal may be suffering from a neurological or muscular disorder. Where the disorder is a neurological disorder, NRG3 is believed to be useful in promoting the development, maintenance, and/or regeneration of neurons *in vivo*, including central (brain and spinal chord), peripheral (sympathetic, parasympathetic, sensory, and enteric neurons), and motoneurons. Accordingly, NRG3 may be utilized in methods for the diagnosis and/or treatment of a variety of neurologic diseases or disorders which affect the nervous system of a mammal, such as a human.

Such diseases or disorders may arise in a patient in whom the nervous system has been

damaged by, *e.g.*, trauma, surgery, stroke, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, or toxic agents. The agent is designed to promote the survival or growth of neurons. For example, NRG3 can be used to promote the survival or growth of motoneurons that are damaged by trauma or surgery. Also, NRG3 can be used to treat motoneuron disorders, such as amyotrophic lateral sclerosis (Lou Gehrig's disease), Bell's palsy, and various conditions involving spinal muscular atrophy, or paralysis. NRG3 can be used to treat human "neurodegenerative disorders", such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, Huntington's chorea, Down's Syndrome, nerve deafness, and Meniere's disease.

Further, NRG3 can be used to treat neuropathy, and especially peripheral neuropathy. "Peripheral neuropathy" refers to a disorder affecting the peripheral nervous system, most often manifested as one or a combination of motor, sensory, sensorimotor, or autonomic neural dysfunction. The wide variety of morphologies exhibited by peripheral neuropathies can each be attributed uniquely to an equally wide number of causes. For example, peripheral neuropathies can be genetically acquired, can result from a systemic disease, or can be induced by a toxic agent. Examples include but are not limited to distal sensorimotor neuropathy, or autonomic neuropathies such as reduced motility of the gastrointestinal tract or atony of the urinary bladder. Examples of neuropathies associated with systemic disease include post-polio syndrome; examples of hereditary neuropathies include Charcot-Marie-Tooth disease, Refsum's disease, Abetalipoproteinemia, Tangier disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, and Dejerine-Sottas syndrome; and examples of neuropathies caused by a toxic agent include those caused by treatment with a chemotherapeutic agent such as vincristine, cisplatin, methotrexate, or 3'-azido-3'-deoxythymidine.

The invention further provides a method for treating a mammal comprising administering a therapeutically effective amount of a NRG3 antagonist to the mammal. The mammal in this latter case is one which could benefit from a reduction in NRG3 levels/biological activity.

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the structure, synthesis, and usage as more fully set forth below. Each reference cited herein is herein incorporated by

reference in its entirety with particular attention to the description of subject matter associated with the context of the citation.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the nucleic acid coding sequence of mouse NRG3 cDNA (mNRG3, SEQ ID NO:1) in which the start (ATG) and stop (TGA) codons of the coding sequence are indicated by underlining.

Fig. 2 shows the nucleic acid coding sequence of human NRG3 cDNA (hNRG3B1, SEQ ID NO:5) in which the start (ATG) and stop (TGA) codons of the coding sequence are indicated by underlining.

Fig. 3 shows the nucleic acid coding sequence of an alternatively spliced form of human NRG3 cDNA (hNRG3B2; SEQ ID NO:22) in which the start (ATG) and stop (TGA) codons of the coding sequence are indicated by underlining.

Figs. 4A - 4B. Fig. 4A shows the deduced amino acid sequences from mouse (mNRG3) and human (hNRG3B1) cDNA as shown in Figs. 1 and 2. Mouse NRG3 deduced amino acid sequence is depicted by SEQ ID NO:2 and human NRG3B1 deduced amino acid sequence is depicted by SEQ ID NO:6. Various putative domains within the amino acid sequences are shown. The EGF-like domain, the N-terminal hydrophobic segment (double underline), the serine/threonine-rich portion, and a predicted transmembrane domain (single underline) are highlighted. Fig. 4B shows the deduced amino acid sequences from hNRG3B1 and hNRG3B2 cDNA as shown in Figs. 2 and 3. Human NRG3B1 deduced amino acid sequence is depicted by SEQ ID NO:6 and human NRG3B2 deduced amino acid sequence is depicted by SEQ ID NO:23. The region of the NRG3 amino acid sequence that differs between the two human sequences is illustrated.

Fig. 5 shows a sequence alignment of the EGF-like domains of human NRG3B1

(hNRG3.egf; SEQ ID NO:4); chicken ARIA (cARIA.egf; SEQ ID NO:9); human amphiregulin (hAR.egf; SEQ ID NO:10); human betacellulin (hBTC.egf; SEQ ID NO:11); human EGF (hEGF.egf; SEQ ID NO:12); human heparin-binding EGF-like growth factor (hHB-EGF.egf; SEQ ID NO:13); human heregulin- α (hHRG α ; SEQ ID NO:14); human heregulin- β (hHRG β .egf; SEQ ID NO:15); human TGF- α (hTGF α .egf; SEQ ID NO:16); and mouse epiregulin (mEPR.egf; SEQ ID NO:17). The sequences were analyzed using Sequence Analysis Programs, Genentech, Inc.

Fig. 6A - 6H are FACS plots demonstrating binding of NRG3^{EGF}.Fc to ErbB4 receptor expressed on the surface of cells. In Figs. 6A-6D, parental K562 cells (Fig. 6A) or K562 cells expressing either ErbB2 receptor (K562^{erbB2} cells; Fig. 6B), ErbB3 receptor (K562^{erbB3} cells; Fig. 6C) or ErbB4 receptor (K562^{erbB4} cells; Fig. 6D) were examined for the expression of corresponding receptors. Cells were incubated with anti-ErbB2 receptor, anti-ErbB3 receptor or anti-ErbB4 receptor antibodies as indicated before PE-conjugated secondary antibody was added. "LOG PE" represents relative fluorescent intensity and "Counts" represents cell numbers. In Figs. 6E-6H, NRG3^{EGF}.Fc is shown by FACS analysis to bind to ErbB4 receptor expressing cells. Parental K562 cells (Fig. 6E), K562^{erbB2} cells (Fig. 6F), K562^{erbB3} cells (Fig. 6G) and K562^{erbB4} cells (Fig. 6H) were incubated with or without NRG3^{EGF}.Fc (containing gD tag) for 1 hour, followed by anti-gD-tag primary antibody and PE-conjugated secondary antibody.

Fig. 7 is a graphical analysis showing competitive inhibition of ¹²⁵I-NRG3^{EGF}.Fc binding to immobilized soluble ErbB4 receptor by NRG3^{EGF}.Fc or NRG^{EGF}. Soluble ErbB4 receptor was immobilized on 96-well plates, and was incubated with various concentrations of unlabeled NRG3^{EGF}.Fc or NRG^{EGF} and constant amount of ¹²⁵I-labeled NRG3^{EGF}.Fc for 1.5 hour at room temperature. The fraction of radioactivity bound over total ¹²⁵I-NRG3^{EGF}.Fc input is plotted against the concentration of competitor. Data of a representative experiment from four independent assays is shown. Error bars indicate standard deviation of quadruplicate samples.

Before the present polypeptides, nucleic acids, vectors, and host cells and processes for

making such are described, it is to be understood that this invention is not limited to the particular compositions of matter and processes described, as such compounds and methods may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

DESCRIPTION OF THE EMBODIMENTS

Definitions

The phrases "novel neuregulin related ligand", "novel NRG3", "novel ErbB4 receptor-specific NRG3" are used interchangeably and refer to a new member of the family of neuregulins, which NRG3 is expressed specifically in the brain and nervous system of the embryo and adults, and to functional derivatives of such native polypeptides.

The term "NRG3" or "neuregulin related ligand" is defined herein to be any polypeptide sequence that possesses at least one biological property (as defined below) of native amino acid sequence NRG3 of SEQ ID NO:2 or 6 (mouse or human, respectively) and additionally includes an alternatively spliced form of human NRG3 having the amino acid sequence of SEQ ID NO:23. This definition encompasses not only the polypeptide isolated from a native NRG3 source such as human MDA-MB-175 cells or from another source, such as another animal species or alternatively spliced forms of NRG3, but also the polypeptide prepared by recombinant or synthetic methods. It also includes variant forms including functional derivatives, allelic variants, naturally occurring isoforms and analogues thereof. Sometimes the NRG3 is "native NRG3" which refers to endogenous NRG3 polypeptide which has been isolated from a mammal. The NRG3 can also be "native sequence NRG3" insofar as it has the same amino acid sequence as a native NRG3 (e.g. mouse (SEQ ID NO:2) or human (SEQ ID NO:6 or SEQ ID NO:23) NRG3 shown in Figs. 4A and 4B). However, "native sequence NRG3" encompasses the polypeptide produced by recombinant or synthetic means. "Mature NRG3" is soluble or secreted NRG3 released from the cell (i.e. lacking an N-terminal hydrophobic sequence). In this context, NRG3 refers to novel NRG3s comprising an EGF-like domain within an extracellular domain, a transmembrane domain and a cytoplasmic domain, with or without a

native signal sequence, and naturally occurring soluble forms of such NRG3s, with or without the initiating methionine, whether purified from native source, synthesized, produced by recombinant DNA technology or by any combination of these and/or other methods. The native NRG3s of the present invention specifically include the murine NRG3, the amino acid sequence of which is shown in Fig. 4 (SEQ. ID. NO:2), and the human NRG3s having the amino acid sequences shown in Fig. 4 (SEQ. ID. NO:6 or SEQ ID NO:23), and fragments or mammalian homologues or alternatively spliced forms of these native ligands. The novel native murine and human NRG3s of the present invention are about 713 and 720 amino acids in length, respectively, and comprise an EGF-like domain, the N-terminal hydrophobic segment, the serine/threonine-rich portion, a predicted transmembrane domain, and a predicted intracellular domain. The boundaries of these domain are indicated in Fig. 4 for the novel murine and human NRG3 sequences.

Optionally, the NRG3 is not associated with native glycosylation. "Native glycosylation" refers to the carbohydrate moieties which are covalently attached to native NRG3 when it is produced in the mammalian cell from which the native NRG3 is derived. Accordingly, human NRG3 produced in a non-human could be described as not being associated with native glycosylation, for example it may be glycosylated other than the native glycosylation. Sometimes, the NRG3 is not associated with any glycosylation whatsoever (e.g. as a result of being produced recombinantly in a prokaryote).

The term "EGF-like domain" refers to an extracellular epidermal growth factor (EGF)-like domain of a polypeptide, preferably a NRG3 polypeptide of the invention. The EGF-like domain is sufficient to bind neuregulin receptors and stimulate cellular responses (Holmes, W.E., et al. (1992) Science 256:1205-1210). Preferably, an EGF-like domain of the NRG3 of the invention has the amino acid sequence of the NRG3s shown in SEQ ID NO:4 (mouse or human NRG3 EGF-like domain), where the EGF-like domain is from about amino acid 284 to about amino acid 332 of human NRG3, and from about amino acid 286 to about amino acid 334 of mouse NRG3. The NRG3 of the invention encompasses a polypeptide encoded by an alternatively spliced form the NRG3 encoding gene, which alternatively spliced form comprises the NRG3 EGF-like domain.

5 The term "ErbB" when used herein refers to any one or more of the mammalian ErbB receptors (i.e. ErbB1 or epidermal growth factor (EGF) receptor; ErbB2 or HER2 receptor; ErbB3 or HER3 receptor; ErbB4 or HER4 receptor; and any other member(s) of this class I tyrosine kinase family to be identified in the future) and "*erbB*" refers to the mammalian *erbB* genes encoding these receptors.

10 The terms "soluble form", "soluble receptor", "soluble NRG3", "soluble NRG3", and grammatical variants thereof, refer to variants of the native or variant NRG3s of the present invention which are devoid of a functional transmembrane domain. In the soluble receptors the transmembrane domain may be deleted, truncated or otherwise inactivated such that they are not capable of cell membrane anchorage. If desired, such soluble forms of the NRG3s of the present invention might additionally have their cytoplasmic domains fully or partially deleted or otherwise inactivated.

15 A "functional derivative" of a polypeptide is a compound having a qualitative biological activity in common with the native polypeptide. Thus, a functional derivative of a native novel NRG3 of the present invention is a compound that has a qualitative biological activity in common with such native NRG3. "Functional derivatives" include, but are not limited to, fragments of native polypeptides from any animal species (including humans), derivatives of native (human and non-human) polypeptides and their fragments, and peptide and non-peptide analogs of native polypeptides, provided that they have a biological activity in common with a respective native polypeptide.

20 As used herein, the term "fragments" refers to regions within the sequence of a mature native polypeptide. Preferably NRG3 fragments will have a consecutive sequence of at least 20, and more preferably at least 50, amino acid residues of the EGF-like domain of NRG3. The preferred fragments have about 30-150 amino acid residues which are identical to a portion of the sequence of NRG3 in SEQ ID NO:2 (from mouse), or in SEQ ID NO:6 or SEQ ID NO:23 (from human). The term "derivative" is used to define amino acid sequence and glycosylation variants, and covalent modifications of a native polypeptide. "Non-peptide analogs" are organic compounds which display substantially the same surface as peptide analogs of the native polypeptides. Thus, the non-peptide analogs of the native novel NRG3s of the present invention

are organic compounds which display substantially the same surface as peptide analogs of the native NRG3s. Such compounds interact with other molecules in a similar fashion as the peptide analogs, and mimic a biological activity of a native NRG3 of the present invention. Preferably, amino acid sequence variants of the present invention retain at least one domain of a native NRG3, preferably an EGF-like domain, or have at least about 60% amino acid sequence identity, more preferably at least about 75 % amino acid sequence identity, and most preferably at least about 90% amino acid sequence identity with a domain of a native NRG3 of the present invention. The amino acid sequence variants preferably show the highest degree of amino acid sequence homology with the EGF-like domain of native NRG3s of the present invention. These are the domains which show the highest percentage amino acid conservation between the novel NRG3s of the present invention and other members of the NRG3 family (see Fig. 4).

The terms "isolated" or "substantially pure" refer to a polypeptide or nucleic acid which is free of other polypeptides or nucleic acids as well as lipids, carbohydrates or other materials with which it is naturally associated. An exception is made for glycosylation wherein sugar moieties are covalently attached to amino acids of the NRG3 polypeptide of the invention. One of ordinary skill in the art can purify a NRG3 polypeptide or nucleic acid encoding the polypeptide using standard techniques appropriate for each type of molecule.

The term "percent amino acid sequence identity" with respect to the NRG3 sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the NRG3 sequence having the deduced amino acid sequence described in Fig. 1, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. N-terminal, C-terminal, or internal extensions, deletions, or insertions into the NRG3 sequence shall be construed as affecting sequence identity or homology.

Another type of NRG3 variant is "chimeric NRG3", which term encompasses a polypeptide comprising full-length NRG3 or a fragment thereof fused or bonded to a heterologous polypeptide. The chimera will normally share at least one biological property with NRG3. Examples of chimeric NRG3s include immunoadhesins and epitope tagged NRG3. In another embodiment, the heterologous polypeptide is thioredoxin, a salvage receptor binding

epitope, cytotoxic polypeptide or enzyme (e.g., one which converts a prodrug to an active drug).

The terms "covalent modification" and "covalent derivatives" are used interchangeably and include, but are not limited to, modifications of a native polypeptide or a fragment thereof with an organic proteinaceous or non-proteinaceous derivatizing agent, fusions to heterologous polypeptide sequences, and post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected sides or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, tyrosyl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton (1983) Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86). Covalent derivatives/modifications specifically include fusion proteins comprising native NRG3 sequences of the present invention and their amino acid sequence variants, such as immunoadhesins, and N-terminal fusions to heterologous signal sequences.

The term "biological activity" in the context of the present invention is defined as the possession of at least one adhesive, regulatory or effector function qualitatively in common with a native polypeptide. Preferred functional derivatives within the scope of the present invention are unified by retaining an EGF-like domain and ErbB4 receptor-specific binding of a native NRG3 of the present invention.

The phrase "activating an ErbB receptor" refers to the act of causing the intracellular kinase domain of an ErbB receptor to phosphorylate tyrosine residues. Generally, this will involve binding of NRG3 to an ErbB4 receptor or ErbB4 receptor homodimer, which binding activates a kinase domain of one or more of the receptors and thereby results in phosphorylation of tyrosine residues in one or more of the receptors, and/or phosphorylation of tyrosine residues

in additional substrate polypeptide(s). ErbB receptor phosphorylation can be quantified using the tyrosine phosphorylation assays described below. It is understood that the NRG3 of the invention may itself be activated by interaction with an ErbB receptor via the intracellular domain of NRG3. Thus, an NRG3-activating ligand that binds to the NRG3 (preferably binding to the extracellular domain, more preferably the EGF-like domain) includes, but is not limited to, a ligand, an antibody, or a receptor. Activation of the NRG3 may be through phosphorylation of the intracellular domain or other like event common to receptor/ligand mediated cell signaling. As a mediator of cell signaling, the NRG3 of the invention is expected to be associated with apoptosis, metabolic signaling, differentiation or cell proliferation.

"Identity" or "homology" with respect to a native polypeptide and its functional derivative is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known in the art. For example, the sequences disclosed herein were analyzed using Sequence Analysis Programs, Genentech, Inc, Inc.

The term "agonist" is used to refer to peptide and non-peptide analogs of the native NRG3s of the present invention and to antibodies specifically binding such native NRG3s provided that they retain at least one biological activity of a native NRG3. Preferably, the agonists of the present invention retain the qualitative EGF-like domain binding recognition properties of the native NRG3 polypeptides.

The term "antagonist" is used to refer to a molecule inhibiting a biological activity of a native NRG3 of the present invention. Preferably, the antagonists herein inhibit the binding of a native NRG3 of the present invention. Preferred antagonists essentially completely block the binding of a native NRG3 to an ErbB4 receptor to which it otherwise binds. A NRG3 "antagonist" is a molecule which prevents, or interferes with, a NRG3 effector function (*e.g.* a molecule which prevents or interferes with binding and/or activation of an ErbB4 receptor by NRG3). Such molecules can be screened for their ability to competitively inhibit ErbB receptor

activation by NRG3 in the tyrosine phosphorylation assay disclosed herein, for example. Preferred antagonists are those which do not substantially interfere with the interaction of other heregulin polypeptides with ErbB receptor(s). Examples of NRG3 antagonists include neutralizing antibodies against NRG3 and antisense polynucleotides against the NRG3 gene.

Ordinarily, the terms "amino acid" and "amino acids" refer to all naturally occurring L- α -amino acids. In some embodiments, however, D-amino acids may be present in the polypeptides or peptides of the present invention in order to facilitate conformational restriction. For example, in order to facilitate disulfide bond formation and stability, a D amino acid cysteine may be provided at one or both termini of a peptide functional derivative or peptide antagonist of the native NRG3s of the present invention. The amino acids are identified by either the single-letter or three-letter designations:

Asp	D	aspartic acid	Ile	I	isoleucine
Thr	T	threonine	Leu	L	leucine
Ser	S	serine	Tyr	Y	tyrosine
Glu	E	glutamic acid	Phe	F	phenylalanine
Pro	P	proline	His	H	histidine
Gly	G	glycine	Lys	K	lysine
Ala	A	alanine	Arg	R	arginine
Cys	C	cysteine	Trp	W	tryptophan
Val	V	valine	Gln	Q	glutamine
Met	M	methionine	Asn	N	asparagine

The term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a native amino acid sequence.

Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native sequence. Immediately adjacent to an amino

acid means connected to either the α -carboxy or α -amino functional group of the amino acid.

Deletional variants are those with one or more amino acids in the native amino acid sequence removed.

"Antibodies (Abs)" and "immunoglobulins (Igs)" are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 Daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia *et al.* (1985) J. Mol. Biol. 186, 651-663; Novotny and Haber (1985) Proc. Natl. Acad. Sci. USA 82:4592-4596).

The light chains of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α , delta, epsilon, γ , and μ , respectively. The subunit structures and three-dimensional configurations of

different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polypeptopic specificity, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein (1975) *Nature* 256:495, or may be made by recombinant DNA methods (see, e.g. U.S. Patent No. 4,816,567 (Cabilly *et al.*) and Mage and Lamoyi (1987) in Monoclonal Antibody Production Techniques and Applications, pp. 79-97, Marcel Dekker, Inc., New York). The monoclonal antibodies may also be isolated from phage libraries generated using the techniques described in McCafferty *et al.* (1990) *Nature* 348:552-554, for example.

"Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab)₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from the complementarity determining regions (CDRs) of the recipient antibody are replaced by residues from the CDRs of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human FR residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or FR sequences. These modifications are made to further refine and

optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR residues are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see: Jones *et al.* (1986) Nature 321:522-525; Reichmann *et al.* (1988) Nature 332:323-329; EP-B-239 400 published 30 September 1987; Presta (1992) Curr. Op. Struct. Biol. 2:593-596; and EP-B-451 216 published 24 January 1996), which references are herein incorporated by reference in their entirety. The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

By "neutralizing antibody" is meant an antibody molecule as herein defined which is able to block or significantly reduce an effector function of native sequence NRG3. For example, a neutralizing antibody may inhibit or reduce the ability of NRG3 to activate an ErbB receptor, preferably an ErbB4 receptor, in the tyrosine phosphorylation assay described herein. The neutralizing antibody may also block the mitogenic activity of NRG3 in the cell proliferation assay disclosed herein.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567 (Cabilly *et al.*; Morrison *et al.* (1984) Proc. Natl. Acad. Sci. USA 81:6851-6855).

In the context of the present invention the expressions "cell", "cell line", and "cell culture" and "host cell" are used interchangeably, and all such designations include progeny. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate

or inadvertent mutations. Mutant progeny that have the same function or biological property, as screened for in the originally transformed cell, are included. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

The terms "replicable expression vector", "expression vector" and "vector" refer to a piece of DNA, usually double-stranded, which may have inserted into it a piece of foreign DNA. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell. The vector is used to transport the foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of the host chromosomal DNA, and several copies of the vector and its inserted (foreign) DNA may be generated. In addition, the vector contains the necessary elements that permit translating the foreign DNA into a polypeptide. Many molecules of the polypeptide encoded by the foreign DNA can thus be rapidly synthesized.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancer.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or a secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods, such as phosphotriester, phosphite, or

phosphoramidite chemistry, using solid phase techniques such as those described in EP 266,032, published 4 May 1988, or via deoxynucleoside H-phosphanate intermediates as described by Froehler *et al.* (1986) Nucl. Acids Res. 14:5399. They are then purified on polyacrylamide gels.

By "solid phase" is meant a non-aqueous matrix to which a reagent of interest (e.g., NRG3 or an antibody thereto) can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149, herein incorporated by reference in its entirety.

The terms "transformation" and "transfection" are used interchangeably herein and refer to the process of introducing DNA into a cell. Following transformation or transfection, the NRG3 DNA may integrate into the host cell genome, or may exist as an extrachromosomal element. If prokaryotic cells or cells that contain substantial cell wall constructions are used as hosts, the preferred methods of transfection of the cells with DNA is the calcium treatment method described by Cohen *et al.* (1972) Proc. Natl. Acad. Sci. U.S.A., 69:2110-2114 or the polyethylene glycol method of Chung *et al.* (1988) Nuc. Acids. Res. 16:3580. If yeast are used as the host, transfection is generally accomplished using polyethylene glycol, as taught by Hinnen (1978) Proc. Natl. Acad. Sci. U.S.A. 75:1929-1933. If mammalian cells are used as host cells, transfection generally is carried out by the calcium phosphate precipitation method, Graham *et al.* (1978) Virology 52:546, Gorman *et al.* (1990) DNA and Protein Eng. Tech. 2:3-10. However, other known methods for introducing DNA into prokaryotic and eukaryotic cells, such as nuclear injection, electroporation, or protoplast fusion also are suitable for use in this invention.

Particularly useful in this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding NRG3. In general, transient expression involves the use of an expression vector that is able to efficiently replicate in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high

levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties.

5 It is further envisioned that the NRG3 of this invention may be produced by homologous recombination, as provided for in WO 91/06667, published 16 May 1991. Briefly, this method involves transforming a cell containing an endogenous NRG3 gene with a homologous DNA, which homologous DNA comprises (a) an amplifiable gene (*e.g.* a gene encoding dihydrofolate reductase (DHFR)), and (b) at least one flanking sequence, having a length of at least about 150
10 base pairs, which is homologous with a nucleotide sequence in the cell genome that is within or in proximity to the gene encoding NRG3. The transformation is carried out under conditions such that the homologous DNA integrates into the cell genome by recombination. Cells having integrated the homologous DNA are then subjected to conditions which select for amplification of the amplifiable gene, whereby the NRG3 gene is amplified concomitantly. The resulting cells are then screened for production of desired amounts of NRG3. Flanking sequences that are in
15 proximity to a gene encoding NRG3 are readily identified, for example, by the method of genomic walking, using as a starting point the nucleotide sequence, or fragment thereof, of mouse NRG3 of Fig. 1 (SEQ ID NO:1), or human NRG3 of Fig. 2 (SEQ ID NO:5) or Fig. 3 (SEQ ID NO:22). DNA encoding the mouse and human NRG3 polypeptides is deposited with
20 the American Type Culture Collection as ATCC _____ (mouse; pLXSN.mNRG3), ATCC _____ (human; pRK5.tk.neo.hNRG3B1), or ATCC _____ (human; pRK5.tk.neo.hNRG3).

The expression "enhancing survival of a cell" refers to the act of increasing the period of existence of a cell, relative to an untreated cell which has not been exposed to NRG3, either *in vitro* or *in vivo*.

25 The phrase "enhancing proliferation of a cell" encompasses the step of increasing the extent of growth and/or reproduction of the cell, relative to an untreated cell, either *in vitro* or *in vivo*. An increase in cell proliferation in cell culture can be detected by counting the number of cells before and after exposure to NRG3 (see the Example below). The extent of proliferation can be quantified via microscopic examination of the degree of confluency. Cell proliferation

can also be quantified by measuring ^3H uptake by the cells.

By "enhancing differentiation of a cell" is meant the act of increasing the extent of the acquisition or possession of one or more characteristics or functions which differ from that of the original cell (*i.e.* cell specialization). This can be detected by screening for a change in the phenotype of the cell (*e.g.* identifying morphological changes in the cell).

"Muscle cells" include skeletal, cardiac or smooth muscle tissue cells. This term encompasses those cells which differentiate to form more specialized muscle cells (*e.g.* myoblasts).

"Isolated NRG3 nucleic acid" is RNA or DNA free from at least one contaminating source nucleic acid with which it is normally associated in the natural source and preferably substantially free of any other mammalian RNA or DNA. The phrase "free from at least one contaminating source nucleic acid with which it is normally associated" includes the case where the nucleic acid is present in the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell. An example of isolated NRG3 nucleic acid is RNA or DNA that encodes a biologically active NRG3 sharing at least 75%, more preferably at least 80%, still more preferably at least 85%, even more preferably 90%, and most preferably 95% sequence identity with the mouse NRG3 shown in Fig. 1 (SEQ ID NO:1), or human NRG3 shown in Fig. 2 (SEQ ID NO:4) or Fig. 3 (SEQ ID NO:22).

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

Hybridization is preferably performed under "stringent conditions" which means (1)

employing low ionic strength and high temperature for washing, for example, 0.015 sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50° C, or (2) employing during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 nM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42° C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6/8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with washes at 42° C in 0.2 x SSC and 0.1% SDS. Yet another example is hybridization using a buffer of 10% dextran sulfate, 2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55 °C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55 °C.

"Immunoadhesins" or "NRG3 - immunoglobulin chimeras" are chimeric antibody-like molecules that combine the functional domain(s) of a binding protein (usually a receptor, a cell-adhesion molecule or a ligand) with the an immunoglobulin sequence. The most common example of this type of fusion protein combines the hinge and Fc regions of an immunoglobulin (Ig) with domains of a cell-surface receptor that recognizes a specific ligand. This type of molecule is called an "immunoadhesin", because it combines "immune" and "adhesion" functions; other frequently used names are "Ig-chimera", "Ig-" or "Fc-fusion protein", or "receptor-globulin."

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. those in need of treatment include those already with the disorder as well as those prone to have the disorder of those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as sheep, dogs, horses, cats, cows, and the like. Preferably, the mammal herein is a human.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate,

citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween™, polyethylene glycol (PEG), and Pluronics™.

General Procedures for the Production of an NRG3 by recombinant DNA technology

A. Identification and isolation of nucleic acid encoding novel neuregulin related ligand, NRG3.

The native NRG3s of the present invention may be isolated from cDNA or genomic libraries. For example, a suitable cDNA library can be constructed by obtaining polyadenylated mRNA from cells known to express the desired NRG3, and using the mRNA as a template to synthesize double stranded cDNA. Suitable sources of the mRNA are embryonic and adult mammalian tissues. mRNA encoding native NRG3s of the present invention is expressed, for example, in adult mammalian, brain, nervous system, heart, muscle, and testis. The gene encoding the novel NRG3s of the present invention can also be obtained from a genomic library, such as a human genomic cosmid library, or a mouse-derived embryonic stem cell (ES) genomic library.

Libraries, either cDNA or genomic, are screened with probes designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal and polyclonal antibodies that recognize and specifically bind to a NRG3 of the invention. For cDNA libraries, suitable probes include carefully selected oligonucleotide probes (usually of about 20-80 bases in length) that encode known or suspected portions of a NRG3 polypeptide from the same or different species, and/or complementary or homologous cDNAs or fragments thereof that encode the same or a similar gene. Appropriate probes for screening genomic DNA libraries include, without limitation, oligonucleotides, cDNAs, or fragments thereof that encode the same or a similar gene, and/or homologous genomic DNAs or

fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in Chapters 10-12 of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, New York, Cold Spring Harbor Laboratory Press, 1989, herein incorporated by reference in its entirety.

5 If DNA encoding a NRG3 of the present invention is isolated by using carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, the oligonucleotide sequences selected as probes should be sufficient in length and sufficiently unambiguous that false positive selections are minimized. The actual nucleotide sequence(s) is/are usually designed based on regions that have the least codon redundancy. The oligonucleotides may be
10 degenerate at one or more positions. The use of degenerate oligonucleotides is of particular importance where a library is screened from a species in which preferential codon usage is not known.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ATP (e.g., $\gamma^{32}\text{P}$) and polynucleotide kinase to radiolabel the 5' end of the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

cDNAs encoding the novel NRG3s can also be identified and isolated by other known techniques of recombinant DNA technology, such as by direct expression cloning, or by using the polymerase chain reaction (PCR) as described in U.S. Patent No. 4,683,195, issued 28 July 1987,
20 in section 14 of Sambrook *et al.*, *supra*, or in Chapter 15 of Current Protocols in Molecular Biology, Ausubel *et al.* eds., Greene Publishing Associates and Wiley-Interscience 1991, which references are herein incorporated by reference in their entirety.

25 Once cDNA encoding a new native ErbB4 receptor-specific NRG3 from one species has been isolated, cDNAs from other species can also be obtained by cross-species hybridization. According to this approach, human or other mammalian cDNA or genomic libraries are probed by labeled oligonucleotide sequences selected from known NRG3 sequences (such as murine or human sequences) in accord with known criteria. Preferably, the probe sequence should be sufficient in length and sufficiently unambiguous that false positives are minimized. Typically, a

³²P-labeled oligonucleotide having about 30 to 50 bases is sufficient, particularly if the oligonucleotide contains one or more codons for methionine or tryptophan. Isolated nucleic acid will be DNA that is identified and separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid. Hybridization is preferably performed under "stringent conditions", as defined herein.

Once the sequence is known, the gene encoding a particular NRG3 can also be obtained by chemical synthesis, following one of the methods described in Engels and Uhlmann, Agnew (1989) Chem. Int. Ed. Engl. 28:716, herein incorporated by reference in its entirety. These methods include triester, phosphite, phosphoramidite and H-phosphonate methods, PCR and other autoprimer methods, and oligonucleotide syntheses on solid supports.

B. Cloning and expression of nucleic acid encoding the novel NRG3s.

Once the nucleic acid encoding a novel NRG3 is available, it is generally ligated into a replicable expression vector for further cloning (amplification of the DNA), or for expression.

Expression and cloning vectors are well known in the art and contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. The selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of the above listed components, the desired coding and control sequences, employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are commonly used to transform *E. coli* cells, e.g. *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are

prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.* (1981) *Nucleic Acids Res.* 9:309 or by the method of Maxam *et al.* (1980) *Methods in Enzymology* 65:499.

The polypeptides of the present invention may be expressed in a variety of prokaryotic and eukaryotic host cells. Suitable prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. A preferred cloning host is *E. coli* 294 (ATCC 31,446) although other gram negative or gram positive prokaryotes such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), *E. coli* W3110 (ATCC 27,325), *Pseudomonas* species, or *Serratia Marcesans* are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for vectors herein. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species and strains are commonly available and useful herein, such as *S. pombe* (Beach and Nurse (1981) *Nature* 290:140), *Kluyveromyces lactis* (Louvencourt *et al.* (1983) *J. Bacteriol.* 737); *yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070), *Trichoderma reesia* (EP 244,234), *Neurospora crassa* (Case *et al.* (1979) *Proc. Natl. Acad. Sci. USA* 76:5259-5263); and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.* (1983) *Biochem. Biophys. Res. Commun.* 112:284-289; Tilburn *et al.* (1983) *Gene* 26:205-221; Yelton *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:1470-1474) and *A. niger* (Kelly and Hynes (1985) *EMBO J.* 4:475-479).

Suitable host cells may also derive from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture, although cells from mammals such as humans are preferred. Examples of invertebrate cells include plants and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melangaster* (fruitfly), and *Bombyx mori* host cells have been identified. See, e.g. Luckow *et al.* (1988) *Bio/Technology* 6:47-55; Miller *et al.*, in *Genetic Engineering*, Setlow, J.K. *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.* (1985) *Nature* 315:592-594. A variety of such viral strains are publicly available, e.g. the

L-1 variant of *Autographa californica* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the NRG3 DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding a NRG3 is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the NRG3 DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.* (1982) J. Mol. Appl. Gen. 1:561. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) is *per se* well known (see for example, Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney cell line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.* (1977) J. Gen. Virol. 36:59); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin (1980) Proc. Natl. Acad. Sci. USA 77:4216); mouse sertolli cells (TM4, Mather (1980) Biol. Reprod. 23:243-251); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.* (1982) Annals N.Y. Acad. Sci. 383:44068); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Particularly useful in the practice of this invention are expression vectors that provide for

the expression in mammalian cells of DNA encoding a novel NRG3 herein. Where transient expression is preferred, expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of a native NRG3 of the invention.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the NRG3s in recombinant vertebrate cell culture are described for example, in Getting *et al.* (1981) *Nature* 293:620-625; Mantel *et al.* (1979) *Nature* 281:40-46; Levinson *et al.*; EP 117,060 and EP 117,058. Particularly useful plasmids for mammalian cell culture expression of the NRG3 polypeptides are pRK5 (EP 307,247), or pSVI6B (PCT Publication No. WO 91/08291).

Other cloning and expression vectors suitable for the expression of the NRG3s of the present invention in a variety of host cells are, for example, described in EP 457,758 published 27 November 1991. A large variety of expression vectors is now commercially available. An exemplary commercial yeast expression vector is pPIC.9 (Invitrogen), while an commercially available expression vector suitable for transformation of *E. coli* cells is PET15b (Novagen).

C. Culturing the Host Cells.

Prokaryote cells used to produced the NRG3s of this invention are cultured in suitable media as describe generally in Sambrook *et al.*, supra.

Mammalian cells can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM, Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace (1979) *Meth. Enzymol.* 58:44; Barnes and Sato (1980) *Anal. Biochem.* 102:255, US 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195 or US Pat. Re. 30,985 may be used as culture media for

the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug) trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH and the like, suitably are those previously used with the host cell selected for cloning or expression, as the case may be, and will be apparent to the ordinary artisan.

The host cells referred to in this disclosure encompass cells in *in vitro* cell culture as well as cells that are within a host animal or plant.

It is further envisioned that the NRG3s of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the particular NRG3.

D. Detecting Gene Amplification and/or Expression.

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas (1980) Proc. Natl. Acad. Sci. USA 77:5201-5205), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ³²P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as a site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to the surface, so that upon the formation of duplex on the surface, the presence of antibody bound

to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. A particularly sensitive staining technique suitable for use in the present invention is described by Hse *et al.* (1980) Am. J. Clin. Pharm. 75:734-738.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any animal. Conveniently, the antibodies may be prepared against a native NRG3 polypeptide, or against a synthetic peptide based on the DNA sequence disclosed herein.

E. Amino Acid Sequence Variants of a Native NRG3.

Amino acid sequence variants of native NRG3s are prepared by methods known in the art by introducing appropriate nucleotide changes into a native NRG3 DNA, or by *in vitro* synthesis of the desired polypeptide. There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. With the exception of naturally-occurring alleles, which do not require the manipulation of the DNA sequence encoding the native NRG3, the amino acid sequence variants of NRG3s are preferably constructed by mutating the DNA, either to arrive at an allele or an amino acid sequence variant that does not occur in nature.

One group of mutations will be created within the extracellular domain or within the EGF-like domain of a novel native mouse or human NRG3 of the present invention (see figure 3 for the delineation of the extracellular domain (SEQ ID NO:3 or SEQ ID NO:7) and EGF-like domain (SEQ ID NO:4) within human or mouse NRG3 amino acid sequences, respectively. Since these domains are believed to be functionally important, alterations such as non-conservative substitutions, insertions and/or deletions in these regions are expected to result in genuine changes in the properties of the native receptor molecules such as in ErbB4 receptor binding and activation. Accordingly, amino acid alterations in this region are also believed to result in variants with properties significantly different from the corresponding native

polypeptides. Non-conservative substitutions within these functionally important domains may result in variants which lose the ErbB4 receptor recognition and binding ability of their native counterparts, or have increased ErbB4 receptor recognition properties, enhanced selectivity, or enhanced activation properties as compared to the corresponding native proteins.

Alternatively or in addition, amino acid alterations can be made at sites that differ in novel NRG3s from various species, or in highly conserved regions, depending on the goal to be achieved. Sites at such locations will typically be modified in series, e.g. by (1) substituting first with conservative choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue or residues, or (3) inserting residues of the same or different class adjacent to the located site, or combinations of options 1-3. One helpful technique for such modifications is called "alanine scanning" (Cunningham and Wells (1989) *Science* 244:1081-1085).

In yet another group of the variant NRG3s of the present invention, one or more of the functionally less significant domains may be deleted or inactivated. For example, the deletion or inactivation of the transmembrane domain yields soluble variants of the native proteins. Alternatively, or in addition, the cytoplasmic domain may be deleted, truncated or otherwise altered.

Naturally-occurring amino acids are divided into groups based on common side chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophobic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Conservative substitutions involve exchanging a member within one group for another member within the same group, whereas non-conservative substitutions will entail exchanging a member of one of these classes for another. Substantial changes in function or immunological identity are made by NRG3 substitutions that are less conservative, i.e. differ more significantly

in their effect on maintaining (a) the structure of the polypeptide backbone in the area of substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the properties of the novel native NRG3s of the present invention will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine. Such substitutions are expected to have their most significant effect when made within the extracellular domain, such as in the EGF-like domain.

Substitutional variants of the novel NRG3s of the present invention also include variants where functionally homologous (having at least about 40%-50% homology) domains of other proteins are substituted by routine methods for one or more of the above-identified domains within the novel NRG3 structure, such as the extracellular domain or EGF-like domain.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Typically, the transmembrane and cytoplasmic domains, or only the transmembrane domains are deleted. However, deletion from the C-terminus to any suitable amino acid N-terminal to the transmembrane region which preserves the biological activity or immunological cross-reactivity of a native NRG3 is suitable. The transmembrane region (TM) of each of the human and mouse NRG3 consensus sequences is shown in Figs. 4A and 4B to range from about amino acid 362 to about amino acid 384 (human SEQ ID NO:6 and SEQ ID NO:23), and about amino acid 360 to about amino acid 382 (mouse SEQ ID NO:2).

A preferred class of substitutional and/or deletional variants of the present invention are those involving a transmembrane region of a novel NRG3 molecule. Transmembrane regions are highly hydrophobic or lipophilic domains that are the proper size to span the lipid bilayer of the cellular membrane. They are believed to anchor the NRG3 in the cell membrane, and allow for

homo- or heteropolymeric complex formation. Inactivation of the transmembrane domain, typically by deletion or substitution of transmembrane domain hydroxylation residues, will facilitate recovery and formulation by reducing its cellular or membrane lipid affinity and improving its aqueous solubility. If the transmembrane and cytoplasmic domains are deleted one avoids the introduction of potentially immunogenic epitopes, whether by exposure of otherwise intracellular polypeptides that might be recognized by the body as foreign or by insertion of heterologous polypeptides that are potentially immunogenic. Inactivation of the membrane insertion function is accomplished by deletion of sufficient residues to produce a substantially hydrophilic hydropathy profile in the transmembrane or by substituting with heterologous residues which accomplish the same result.

A principle advantage of the transmembrane inactivated variants of the NRG3s of the present invention is that they may be secreted into the culture medium of recombinant hosts. These variants are soluble in body fluids such as blood and do not have an appreciable affinity for cell membrane lipids, thus considerably simplifying their recovery from recombinant cell culture. As a general proposition, such soluble variants will retain a functional extracellular domain or fragment thereof, will not have a functional transmembrane domain, and preferably will not have a functional cytoplasmic domain.

For example, the transmembrane domain may be substituted by any amino acid sequence, e.g. a random or predetermined sequences of about 5 to 50 serine, threonine, lysine, arginine, glutamine, aspartic acid and like hydrophilic residues, which altogether exhibit a hydrophilic hydropathy profile. Like the deletional (truncated) soluble variants, these variants are secreted into the culture medium of recombinant hosts.

Amino acid insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e. insertions within the novel NRG3 amino acid sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5 residues, more preferably 1 to 3 residues. An example of a terminal insertion includes fusion of a heterologous N-terminal signal sequence to the N-terminus of the NRG3 molecule to facilitate the secretion of the mature NRG3 or a fragment thereof from

recombinant host cells. Such signal sequences will generally be obtained from, and thus be homologous to, a signal sequence of the intended host cell species. Suitable sequences include STII or Ipp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

5 Other insertional variants of the native NRG3 molecules include the fusion of the N- or C-terminus of the NRG3 molecule to immunogenic polypeptides, e.g. bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli* trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin regions (preferably immunoglobulin constant regions), albumin, or ferritin, as described in WO 89/02922 published on 6 April 1989.

10 Further insertional variants are immunologically active derivatives of the novel NRG3s, which comprise the EGF-like domain and a polypeptide containing an epitope of an immunologically competent extraneous polypeptide, i.e. a polypeptide which is capable of eliciting an immune response in the animal to which the fusion is to be administered or which is capable of being bound by an antibody raised against an extraneous polypeptide. Typical examples of such immunologically competent polypeptides are allergens, autoimmune epitopes, or other potent immunogens or antigens recognized by pre-existing antibodies in the fusion recipient, including bacterial polypeptides such as trpLE, β -galactosidase, viral polypeptides such as herpes gD protein, and the like.

15 20 Immunogenic fusions are produced by cross-linking *in vitro* or by culture of cells transformed with recombinant DNA encoding an immunogenic polypeptide. It is preferable that the immunogenic fusion be one in which the immunogenic sequence is joined to or inserted into a novel NRG3 molecule or fragment thereof by one or more peptide bonds. These products therefore consist of a linear polypeptide chain containing the NRG3 epitope and at least one epitope foreign to the NRG3. It will be understood that it is within the scope of this invention to introduce the epitopes anywhere within a NRG3 molecule of the present invention or a fragment thereof. These immunogenic insertions are particularly useful when formulated into a pharmacologically acceptable carrier and administered to a subject in order to raise antibodies against the NRG3 molecule, which antibodies in turn are useful as diagnostics, in tissue-typing,

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or in purification of the novel NRG3s by standard immunoaffinity techniques. Alternatively, in the purification of the NRG3s of the present invention, binding partners for the fused extraneous polypeptide, e.g. antibodies, receptors or ligands, are used to adsorb the fusion from impure admixtures, after which the fusion is eluted and, if desired, the novel NRG3 is recovered from the fusion, e.g. by enzymatic cleavage.

Since it is often difficult to predict in advance the characteristics of a variant NRG3, it will be appreciated that some screening will be needed to select the optimum variant. Such screening includes, but is not limited to, arrays of ErbB4 receptor binding.

After identifying the desired mutation(s), the gene encoding a NRG3 variant can, for example, be obtained by chemical synthesis as described herein. More preferably, DNA encoding a NRG3 amino acid sequence variant is prepared by site-directed mutagenesis of DNA that encodes an earlier prepared variant or a nonvariant version of the NRG3. Site-directed (site-specific) mutagenesis allows the production of NRG3 variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the techniques of site-specific mutagenesis are well known in the art, as exemplified by publications such as, Edelman *et al.* (1983) DNA 2:183. As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al.*, Third Cleveland Symposium on Macromolecules and Recombinant DNA, A. Walton, ed., Elsevier, Amsterdam (1981). This and other phage vectors are commercially available and their use is well known to those skilled in the art. A versatile and efficient procedure for the construction of oligodeoxyribonucleotide directed site-specific mutations in DNA fragments using M13-derived vectors was published by Zoller, M.J. and Smith, M. (1982) Nucleic Acids Res. 10:6487-6500). Also, plasmid vectors that contain a single-stranded phage origin of replication (Veira *et al.* (1987) Meth. Enzymol. 153:3)

may be employed to obtain single-stranded DNA. Alternatively, nucleotide substitutions are introduced by synthesizing the appropriate DNA fragment *in vitro*, and amplifying it by PCR procedures known in the art.

The PCR amplification technique may also be used to create amino acid sequence variants of a novel NRG3. In a specific example of PCR mutagenesis, template plasmid DNA (1 µg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide triphosphates and is included in the GeneAmp^R kits (obtained from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA), and 25 pmole of each oligonucleotide primer, to a final volume of 50 µl. The reaction mixture is overlaid with 35 µl mineral oil. The reaction is denatured for 5 minutes at 100°C, placed briefly on ice, and then 1 µl *Thermus aquaticus* (*Taq*) DNA polymerase (5 units/l), purchased from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (Perkin-Elmer Cetus) programmed as follows: (as an example)

2 min. 55°C,
30 sec. 72°C, then 19 cycles of the following:
30 sec. 94°C,
30 sec. 55°C, and
30 sec. 72°C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50 vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to appropriate treatments for insertion into a vector.

Cassette mutagenesis is another method useful for preparing variants and is based on the technique described by Wells *et al.* (1985) *Gene* 34:315.

Additionally, the so-called phagemid display method may be useful in making amino acid sequence variants of native or variant NRG3s or their fragments. This method involves

1) constructing a replicable expression vector comprising a first gene encoding a receptor to be

mutated, a second gene encoding at least a portion of a natural or wild-type phage coat protein wherein the first and second genes are heterologous, and a transcription regulatory element operably linked to the first and second genes, thereby forming a gene fusion encoding a fusion protein; 2) mutating the vector at one or more selected positions within the first gene thereby forming a family of related plasmids; 3) transforming suitable host cells with the plasmids; 4) infecting the transformed host cells with a helper phage having a gene encoding the phage coat protein; 5) culturing the transformed infected host cells under conditions suitable for forming recombinant phagemid particles containing at least a portion of the plasmid and capable of transforming the host, the conditions adjusted so that no more than a minor amount of phagemid particles display more than one copy of the fusion protein on the surface of the particle; 6) contacting the phagemid particles with a suitable antigen so that at least a portion of the phagemid particles bind to the antigen; and 7) separating the phagemid particles that bind from those that do not. Steps 4 through 7 can be repeated one or more times. Preferably in this method the plasmid is under tight control of the transcription regulatory element, and the culturing conditions are adjusted so that the amount or number of phagemid particles displaying more than one copy of the fusion protein on the surface of the particle is less than about 1%. Also, preferably, the amount of phagemid particles displaying more than one copy of the fusion protein is less than 10% of the amount of phagemid particles displaying a single copy of the fusion protein. Most preferably, the amount is less than 20%. Typically in this method, the expression vector will further contain a secretory signal sequence fused to the DNA encoding each subunit of the polypeptide and the transcription regulatory element will be a promoter system. Preferred promoter systems are selected from *lac Z*, λ_{PL} , *tac*, T7 polymerase, tryptophan, and alkaline phosphatase promoters and combinations thereof. Also, normally the method will employ a helper phage selected from M13K07, M13R408, M13-VCS, and Phi X 174. The preferred helper phage is M13K07, and the preferred coat protein is the M13 Phage gene III coat protein. The preferred host is *E. coli*, and protease-deficient strains of *E. coli*.

Further details of the foregoing and similar mutagenesis techniques are found in general textbooks, such as, for example, Sambrook *et al.*, *supra*, and Current Protocols in Molecular Biology, Ausubel *et al.* eds., *supra*.

F. Glycosylation variants.

Glycosylation variants are included within the scope of the present invention. They include variants completely lacking in glycosylation (unglycosylated), variants having at least one less glycosylated site than the native form (deglycosylated) as well as variants in which the glycosylation has been changed. Included are deglycosylated and unglycosylated amino acid sequences variants, deglycosylated and unglycosylated native NRG3s or fragments thereof and other glycosylation variants. For example, substitutional or deletional mutagenesis may be employed to eliminate the N- or O-linked glycosylation sites in the a native or variant NRG3 of the present invention, e.g. the asparagine residue may be deleted or substituted for another basic residue such as lysine or histidine. Alternatively, flanking residues making up the glycosylation site may be substituted or deleted, even though the asparagine residues remain unchanged, in order to prevent glycosylation by eliminating the glycosylation recognition site. Where the preferred NRL variant is the EGF-like domain of NRG3, the fragment is preferably unglycosylated.

Additionally, unglycosylated NRG3s which have the glycosylation sites of a native molecule may be produced in recombinant prokaryotic cell culture because prokaryotes are incapable of introducing glycosylation into polypeptides.

Glycosylation variants may be produced by appropriate host cells or by *in vitro* methods. Yeast and insect cells, for example, introduce glycosylation which varies significantly from that of mammalian systems. Similarly, mammalian cells having a different species (e.g. hamster, murine, porcine, bovine or ovine), or tissue origin (e.g. lung, liver, lymphoid, mesenchymal or epidermal) than the source of the NRG3 are routinely screened for the ability to introduce variant glycosylation as characterized for example by elevated levels of mannose or variant ratios of mannose, fucose, sialic acid, and other sugars typically found in mammalian glycoproteins. *In vitro* processing of the NRG3 typically is accomplished by enzymatic hydrolysis, e.g. neuraminidase digestion.

G. Covalent Modifications.

Covalent modifications of the novel NRG3s of the present invention are included within the scope of the invention. Such modifications are traditionally introduced by reacting targeted amino acid residues of the NRG3s with an organic derivatizing agent that is capable of reacting with selected amino acid side chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays of the NRG3, or for the preparation of anti-NRG3 antibodies for immunoaffinity purification of the recombinant. For example, complete inactivation of the biological activity of the protein after reaction with ninhydrin would suggest that at least one arginyl or lysyl residue is critical for its activity, whereafter the individual residues which were modified under the conditions selected are identified by isolation of a peptide fragment containing the modified amino acid residue. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

Derivatization with bifunctional agents is useful for preparing intramolecular aggregates of the NRG3s with polypeptides as well as for cross-linking the NRG3 polypeptide to a water insoluble support matrix or surface for use in assays or affinity purification. In addition, a study of interchain cross-links will provide direct information on conformational structure. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, homobifunctional imidoesters, and bifunctional maleimides. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates which are capable of forming cross-links in the presence of light. Alternatively, reactive water insoluble matrices such as cyanogen bromide activated carbohydrates and the systems reactive substrates described in U.S. Patent Nos. 3,959,642; 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; 4,055,635; and 4,330,440 are employed for protein immobilization and cross-linking.

Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminy and aspariginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively,

these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, threonyl or tyrosyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton (1983) *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86).

Further derivatives of the NRG3s herein are the so called "immunoadhesins", which are chimeric antibody-like molecules combining the functional domain(s) of a binding protein (usually a receptor, a cell-adhesion molecule or a ligand) with the an immunoglobulin sequence. The most common example of this type of fusion protein combines the hinge and Fc regions of an immunoglobulin (Ig) with domains of a cell-surface receptor that recognizes a specific ligand. This type of molecule is called an "immunoadhesin", because it combines "immune" and "adhesion" functions; other frequently used names are "Ig-chimera", "Ig-" or "Fc-fusion protein", or "receptor-globulin."

Immunoadhesins reported in the literature include, for example, fusions of the T cell receptor (Gascoigne *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:2936-2940); CD4 (Capon *et al.* (1989) *Nature* 337:525-531; Traunecker *et al.* (1989) *Nature* 339:68-70; Zettmeissl *et al.* (1990) *DNA Cell Biol. USA* 9:347-353; Byrn *et al.* (1990) *Nature* 344:667-670); L-seNRG3 (homing receptor) (Watson *et al.* (1990) *J. Cell. Biol.* 110:2221-2229); Watson *et al.* (1991) *Nature* 349:164-167); E-seNRG3 (Mulligan *et al.* (1993) *J. Immunol.* 151:6410-17; Jacob *et al.* (1995) *Biochemistry* 34:1210-1217); P-seNRG3 (Mulligan *et al.*, *supra*; Hollenbaugh *et al.* (1995) *Biochemistry* 34:5678-84); ICAM-1 (Stauton *et al.* (1992) *J. Exp. Med.* 176:1471-1476; Martin *et al.* (1993) *J. Virol.* 67:3561-68; Roep *et al.* (1994) *Lancet* 343:1590-93); ICAM-2 (Damle *et al.* (1992) *J. Immunol.* 148:665-71); ICAM-3 (Holness *et al.* (1995) *J. Biol. Chem.* 270:877-84); LFA-3 (Kanner *et al.* (1992) *J. Immunol.* 148:23-29); L1 glycoprotein (Doherty *et al.* (1995) *Neuron* 14:57-66); TNF-R1 (Ashkenazi *et al.*, (1991) *Proc. Natl. Acad. Sci. USA* 88:10535-539); Lesslauer *et al.* (1991) *Eur. J. Immunol.* 21:2883-86; Peppel *et al.* (1991) *J. Exp. Med.* 174:1483-1489); TNF-R2 (Zack *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:2335-39; Wooley *et al.* (1993) *J. Immunol.* 151:6602-07); CD44 (Aruffo *et al.* (1990) *Cell* 61:1303-1313); CD28 and B7

(Linsley *et al.* (1991) J. Exp. Med. 173:721-730); CTLA-4 (Lisley *et al.* (1991) J. Exp. Med. 174:561-569); CD22 (Stamenkovic *et al.* (1991) Cell 66:1133-1144); NP receptors (Bennett *et al.* (1991) J. Biol. Chem. 266:23060-23067); IgE receptor α (Ridgway and Gorman (1991) J. Cell. Biol. 115:1448 abstr.); IFN- γ R α - and β -chain (Marsters *et al.* (1995) Proc. Natl. Acad. Sci. USA 92:5401-05); trk-A, -B, and -C (Shelton *et al.* (1995) J. Neurosci. 15:477-91); IL-2 (Landolfi (1991) J. Immunol. 146:915-19); IL-10 (Zheng *et al.* (1995) J. Immunol. 154:5590-5600).

The simplest and most straightforward immunoaderhin design combines the binding region(s) of the 'adhesin' protein with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the NRG3-immunoglobulin chimeras of the present invention, nucleic acid encoding the desired NRG3 polypeptide will be fused at the C-terminus of the desired sequence to the N-terminus of a nucleic acid sequence encoding an immunoglobulin constant domain sequence, however fusion to the N-terminus of the desired NRG3 sequence is also possible. Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the NRG3-immunoglobulin chimeras.

In a preferred embodiment, the sequence of a native, mature NRG3 polypeptide, or a soluble form thereof such as a (transmembrane domain-inactivated or EGF-like domain polypeptide) form thereof, is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. IgG-1. It is possible to fuse the entire heavy chain constant region to the NRG3 sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114 (Kobet *et al.*, *supra*), or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the NRG3 sequence (full length or

soluble) is fused to the hinge region and CH2 and CH3 or CH1, hinge, CH2 and CH3 domains of an IgG-1, IgG-2, or IgG-3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

In some embodiments, the NRG3-immunoglobulin chimeras are assembled as multimers, and particularly as homo-dimers or -tetramers (WO 91/08298). Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each four unit may be the same or different.

Various exemplary assembled NRG3-immunoglobulin chimeras within the scope of the invention are schematically diagrammed below:

- (a) AC_L-AC_L ;
- (b) $AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, \text{ or } V_LC_L-AC_H]$;
- (c) $AC_L-AC_H-[AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ or } V_LC_L-V_HC_H]$;
- (d) $AC_L-V_HC_H-[AC_H, \text{ or } AC_L-V_HC_H, \text{ or } V_LC_L-AC_H]$;
- (e) $V_LC_L-AC_H-[AC_L-V_HC_H, \text{ or } V_LC_L-AC_H]$; and
- (f) $[A-Y]_n-[V_LC_L-V_HC_H]_2$,

wherein

each A represents identical or different novel NRG3 polypeptide amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interest of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed as

being present in the ordinary locations which they occupy in the immunoglobulin molecules.

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an NRG3-immunoglobulin heavy chain fusion polypeptide, or directly fused to the NRG3 polypeptide. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the NRG3-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Patent No. 4,816,567 issued 28 March 1989.

In a preferred embodiment, the immunoglobulin sequences used in the construction of the immunoadhesins of the present invention are from an IgG immunoglobulin heavy chain constant domain. For human immunoadhesins, the use of human IgG-1 and IgG-3 immunoglobulin sequences is preferred. A major advantage of using IgG-1 is that IgG-1 immunoadhesins can be purified efficiently on immobilized protein A. In contrast, purification of IgG-3 requires protein G, a significantly less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the IgG-3 hinge is longer and more flexible, so it can accommodate larger 'adhesin' domains that may not fold or function properly when fused to IgG-1. While IgG immunoadhesins are typically mono- or bivalent, other Ig subtypes like IgA and IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. Multimeric immunoadhesins are advantageous in that they can bind their respective targets with greater avidity than their IgG-based counterparts. Reported examples of such structures are CD4-IgM (Traunecker *et al.*, supra); ICAM-IgM (Martin *et al.* (1993) J. Virol. 67:3561-68); and CD2-IgM (Arulanandam *et al.* (1993) J. Exp. Med. 177:1439-50).

For NRG3-Ig immunoadhesins, which are designed for *in vivo* application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG-1, IgG-2 and IgG-4 all have *in vivo* half-lives of 21 days, their relative potencies at activating the complement system are different. IgG-4 does not activate

complement, and IgG-2 is significantly weaker at complement activation than IgG-1. Moreover, unlike IgG-1, IgG-2 does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG-3 is optimal for complement activation, its *in vivo* half-life is approximately one third of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically-defined allotypes are preferred. For example, IgG-1 has only four serologically-defined allotypic sites, two of which (G1m and 2) are located in the Fc region; and one of these sites G1m1, is non-immunogenic. In contrast, there are 12 serologically-defined allotypes in IgG-3, all of which are in the Fc region; only three of these sites (G3m5, 11 and 21) have one allotype which is nonimmunogenic. Thus, the potential immunogenicity of a $\gamma 3$ immunoadhesin is greater than that of a $\gamma 1$ immunoadhesin.

NRG3-Ig immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the NRG3 portion in-frame to an Ig cDNA sequence. However, fusion to genomic Ig fragments can also be used (see, e.g. Gascoigne *et al.* (1987) Proc. Natl. Acad. Sci. USA 84:2936-2940; Aruffo *et al.* (1990) Cell 61:1303-1313; Stamenkovic *et al.* (1991) Cell 66:1133-1144). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequence from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques.

Other derivatives of the novel NRG3s of the present invention, which possess a longer half-life than the native molecules comprise the NRG3, NRG3 fragment (such as the EGF-like domain) or a NRG3-immunoglobulin chimera, covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e., a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are useful, as are polymers which are isolated from native sources. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol (PEG); polyelkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates;

carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including
5 homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextrane sulfate, dextran, dextrans, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; heparin or heparon. The polymer prior to cross-linking need not be, but preferably is, water soluble, but the final conjugate must be water
10 soluble. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if it is intended to be administered by such routes.

Preferably the polymer contains only a single group which is reactive. This helps to avoid cross-linking of protein molecules. However, it is within the scope herein to optimize reaction conditions to reduce cross-linking, or to purify the reaction products through gel
15 filtration or chromatographic sieves to recover substantially homogenous derivatives.

The molecular weight of the polymer may desirably range from about 100 to 500,000, and preferably is from about 1,000 to 20,000. The molecular weight chosen will depend upon the nature of the polymer and the degree of substitution. In general, the greater the
20 hydrophilicity of the polymer and the greater the degree of substitution, the lower the molecular weight that can be employed. Optimal molecular weights will be determined by routine experimentation.

The polymer generally is covalently linked to the novel NRG3, NRG3 fragment or to the NRG3-immunoglobulin chimeras through a multifunctional crosslinking agent which reacts with
25 the polymer and one or more amino acid or sugar residues of the NRG3 or NRG3-immunoglobulin chimera to be linked. However, it is within the scope of the invention to directly crosslink the polymer by reacting a derivatized polymer with the hybrid, or *vice versa*.

The covalent crosslinking site on the NRG3 or NRG3-Ig includes the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino,

carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the hybrid without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent binding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, succinimidyl active esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylchloroformate or P-nitrophenylchloroformate activated PEG.) Carboxyl groups are derivatized by coupling PEG-amine using carbodiimide.

Polymers are conjugated to oligosaccharide groups by oxidation using chemicals, e.g. metaperiodate, or enzymes, e.g. glucose or galactose oxidase, (either of which produces the aldehyde derivative of the carbohydrate), followed by reaction with hydrazide or amino derivatized polymers, in the same fashion as is described by Heitzmann *et al.* (1974) P.N.A.S. 71:3537-41 or Bayer *et al.* (1979) *Methods in Enzymology* 62:310, for the labeling of oligosaccharides with biotin or avidin. Further, other chemical or enzymatic methods which have been used heretofore to link oligosaccharides are particularly advantageous because, in general, there are fewer substitutions than amino acid sites for derivatization, and the oligosaccharide products thus will be more homogenous. The oligosaccharide substituents also are optionally modified by enzyme digestion to remove sugars, e.g. by neuraminidase digestion, prior to polymer derivatization.

The polymer will bear a group which is directly reactive with an amino acid side chain, or the N- or C-terminus of the polypeptide linked, or which is reactive with the multifunctional cross-linking agent. In general, polymers bearing such reactive groups are known for the preparation of immobilized proteins. In order to use such chemistries here, one should employ a water soluble polymer otherwise derivatized in the same fashion as insoluble polymers heretofore employed for protein immobilization. Cyanogen bromide activation is a particularly useful procedure to employ in crosslinking polysaccharides.

"Water soluble" in reference to the starting polymer means that the polymer or its reactive intermediate used for conjugation is sufficiently water soluble to participate in a derivatization reaction. "Water soluble" in reference to the polymer conjugate means that the conjugate is

soluble in physiological fluids such as blood.

The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the protein, whether all or a fragment of the protein is used, whether the protein is a fusion with a heterologous protein (e.g. a NRG3-immunoglobulin chimera), the molecular weight, hydrophilicity and other characteristics of the polymer, and the particular protein derivatization sites chosen. In general, the conjugate contains about from 1 to 10 polymer molecules, while any heterologous sequence may be substituted with an essentially unlimited number of polymer molecules so long as the desired activity is not significantly adversely affected. The optimal degree of cross-linking is easily determined by an experimental matrix in which the time, temperature and other reaction conditions are varied to change the degree of substitution, after which the ability of the conjugates to function in the desired fashion is determined.

The polymer, e.g. PEG, is cross-linked by a wide variety of methods known *per se* for the covalent modification of proteins with nonproteinaceous polymers such as PEG. Certain of these methods, however, are not preferred for the purposes herein. Cyanuronic chloride chemistry leads to many side reactions, including protein cross-linking. In addition, it may be particularly likely to lead to inactivation of proteins containing sulfhydryl groups. Carbonyl diimidazole chemistry (Beauchamp *et al.* (1983) *Anal Biochem.* 131:25-33) requires high pH (>8.5), which can inactivate proteins. Moreover, since the "activated PEG" intermediate can react with water, a very large molar excess of "activated PEG" over protein is required. The high concentrations of PEG required for the carbonyl diimidazole chemistry also led to problems in purification, as both gel filtration chromatography and hydrophilic interaction chromatography are adversely affected. In addition, the high concentrations of "activated PEG" may precipitate protein, a problem that *per se* has been noted previously (Davis, U.S. Patent No. 4,179,337). On the other hand, aldehyde chemistry (Royer, U.S. Patent No. 4,002,531) is more efficient since it requires only a 40-fold molar excess of PEG and a 1-2 hr incubation. However, the manganese dioxide suggested by Royer for preparation of the PEG aldehyde is problematic "because of the pronounced tendency of PEG to form complexes with metal-based oxidizing agents" (Harris *et al.* (1984) *J. Polym. Sci. Polym. Chem. Ed.* 22:341-52). The use of a Moffatt oxidation, utilizing

DMSO and acetic anhydride, obviates this problem. In addition, the sodium borohydride suggested by Royer must be used at high pH and has a significant tendency to reduce disulfide bonds. In contrast, sodium cyanoborohydride, which is effective at neutral pH and has very little tendency to reduce disulfide bonds is preferred.

5 The long half-life conjugates of this invention are separated from the unreacted starting materials by gel filtration. Heterologous species of the conjugates are purified from one another in the same fashion. The polymer also may be water-insoluble, as a hydrophilic gel.

10 The novel NRG3s may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, in colloidal drug delivery systems (e.g. liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th Edition, Osol, A., Ed. (1980).

H. Antibody preparation.

(i) Polyclonal antibodies

15 Polyclonal antibodies to a NRG3, or fragment thereof (such as the EGF-like domain) of the present invention generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the NRG3 and an adjuvant. It may be useful to conjugate the NRG3 or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g. keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

20 Animals are immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freud's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freud's complete adjuvant by subcutaneous injection at multiple sites. 7 to 14 days later the animals are bled and the serum is assayed for anti-NRG3 antibody titer. Animals are boosted until the titer plateaus. Preferably,

the animal boosted with the conjugate of the same NRG3, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

5 (ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. For example, the anti-NRG3 monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler and Milstein (1975) *Nature* 256:495, or may be made by recombinant DNA methods (Cabilly, *et al.*, U.S. Pat. No. 4,816,567).

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, Morrison, *et al.* (1984) *Proc. Nat. Acad. Sci.* 81:6851, or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of a NRG3 monoclonal antibody herein.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for a NRG3 and another antigen-

combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

For diagnostic applications, the antibodies of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; biotin; radioactive isotopic labels, such as, e.g., ^{125}I , ^{32}P , ^{14}C , or ^3H , or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter, *et al.* (1962) *Nature* 144:945; David, *et al.* (1974) *Biochemistry* 13:1014; Pain, *et al.* (1981) *J. Immunol. Meth.* 40:219; and Nygren (1982) *J. Histochem. and Cytochem.* 30:407.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc., 1987).

(iii) Humanized antibodies

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.* (1986) *Nature* 321:522-525; Riechmann *et al.* (1988) *Nature* 332:323-327; Verhoeyen *et al.* (1988) *Science* 239:1534-1536), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a

human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly, *supra*), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

It is important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e. the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding. For further details see PCT/US93/07832, which is a continuation-in-part of PCT/US92/05126, which references are herein incorporated by reference in their entirety.

Alternatively, it is now possible to produce transgenic animals (e.g. mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g. Jakobovits *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:2551-255; Jakobovits *et al.* (1993) Nature 362:255-258.

(iv) Bispecific antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for a NRG3 of the present invention the other one is for any other antigen, for example, another member of the NRG3 family. Such constructs can also be referred to as
5 bispecific immunoadhesins.

Traditionally, the recombinant production of bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Millstein and Cuello (1983) Nature 305:537-539). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas)
10 produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in PCT application publication No. WO 93/08829 (published 13 May 1993), and in Traunecker *et al.* (1991) EMBO 10:3655-3659. This problem may be overcome by selecting a
15 common light chain for each arm of the bispecific antibody such that binding specificity of each antibody is maintained, as disclosed in US Application Serial No. 08/850058, filed May 5, 1997.

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain
20 constant domain, comprising at least part of the hinge, and second and third constant regions of an immunoglobulin heavy chain (CH2 and CH3). It is preferred to have the first heavy chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are cotransfected
25 into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or

when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in PCT application WO 94/04690 published 3 March 1994.

For further details of generating bispecific antibodies see, for example, Suresh *et al.* (1986) *Methods in Enzymology* 121:210.

(v) Heteroconjugate antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (PCT application publication Nos. WO 91/00360 and WO 92/200373; EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

I. Diagnostic Kits and Articles of Manufacture.

Since the invention provides a diagnostic assay (*i.e.* for detecting neurological disorders and for detecting the presence of NRG3 in a sample using antibodies or DNA markers) as a matter of convenience, the reagents for these assays can be provided in a kit, *i.e.*, a packaged combination of reagents, for combination with the sample to be tested. The components of the kit will normally be provided in predetermined ratios. Thus, a kit may comprise the antibody or NRG3 (DNA or polypeptide or fragment thereof) labeled directly or indirectly with a suitable label. Where the detectable label is an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.* a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers and the

like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration. The kit also suitably includes instructions for carrying out the bioassay.

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the neurological disorders described herein is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is NRG3 or an agonist or antagonist thereof. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

J. Peptide and non-peptide analogs.

Peptide analogs of the NRG3s of the present invention are modeled based upon the three-dimensional structure of the native polypeptides. Peptides may be synthesized by well known techniques such as the solid-phase synthetic techniques initially described in Merrifield (1963) J. Am. Chem. Soc. 15:2149-2154. Other peptide synthesis techniques are, for examples, described in Bodanszky *et al.*, Peptide Synthesis, John Wiley & Sons, 2nd Ed., 1976, as well as in other reference books readily available for those skilled in the art. A summary of peptide synthesis techniques may be found in Stuart and Young, Solid Phase Peptide Synthelia, Pierce Chemical Company, Rockford, IL (1984). Peptides may also be prepared by recombinant DNA technology, using a DNA sequence encoding the desired peptide.

In addition to peptide analogs, the present invention also contemplates non-peptide (e.g. organic) compounds which display substantially the same surface as the peptide analogs of the present invention, and therefore interact with other molecules in a similar fashion.

5 K. Uses of the NRG3s.

Amino acid sequence variants of the native NRG3s of the present invention may be employed therapeutically to compete with the normal binding of the native proteins to their receptor, ErbB4. The NRG3 amino acid sequence variants are, therefore, useful as competitive inhibitors of the biological activity of native NRG3s.

10 Native NRG3s and their amino acid sequence variants are useful in the identification and purification of the native ErbB4 receptor. The purification is preferably performed by immunoadhesins comprising a NRG3 amino acid sequence retaining the qualitative ability of a native NRG3 of the present invention to recognize its native ErbB4 receptor.

15 The native NRG3s of the present invention are further useful as molecular markers of the tissues in which the ErbB4 receptor is expressed.

20 Furthermore, the NRG3s, preferably the EGF-like domain of the NRG3 of the present invention, provide valuable sequence motifs which can be inserted or substituted into other native members of the NRG3 family of molecules, such as the heregulins. The alteration of these native proteins by the substitution or insertion of sequences from the novel NRG3s of the present invention can yield variant molecules with altered biological properties, such as receptor binding affinity or receptor specificity. For example, one or more NRG3 domains of another member of the NRG3 family may be entirely or partially replaced by NRG3 domain sequences derived from the NRG3s of the present invention. Similarly, EGF-like domain sequences from the NRG3s herein may be substituted or inserted into the amino acid sequences of other NRG3s.

25 Nucleic acid encoding the NRG3s of the present invention is also useful in providing hybridization probes for searching cDNA and genomic libraries for the coding sequence of other NRG3s.

 Additionally, NRG3s of the invention are useful in kits for the diagnosis of disease related to NRG3 and for methods of detecting the presence or absence of NRG3 in a sample,

such as a body fluid, as described herein.

Binding and activation of the ErbB4 receptor by NRG3 is expected to mediate such physiological responses in cells expressing the ErbB4 receptor as cell growth, cell proliferation, and cell differentiation particularly in neural tissue. As a result, mammalian NRG3, or an ErbB4 receptor binding and activating fragment thereof, is useful in the treatment of diseases in which neural cell growth, proliferation and/or differentiation alleviate symptoms of the disease. The NRG3 may be the full length amino acid sequence of the murine NRG3 (SEQ ID NO:2) or the human NRG3s (SEQ ID NO:6 or SEQ ID NO:23); the full length amino acid sequence from another mammalian species having at least approximately 75% homology to the murine and human NRG3 at the amino acid level, preferably about 90% amino acid sequence homology in the EGF-like binding domain; and an amino acid sequence comprising the EGF-like domain of NRG3, which sequence binds to the ErbB4 receptor. Where the NRG3 or ErbB4 receptor binding fragment is agonist, the NRG3 or fragment binds to and activates ErbB4 receptor. Where the NRG3 or fragment is an antagonist, the NRG3 or fragment binds to but does not activate ErbB4 receptor, thereby preventing activation by the naturally occurring NRG3 or agonist.

Diseases treatable by administration of NRG3 or an agonist thereof (such as a polypeptide comprising an NRG3 EGF-like domain) include, but are not limited to, disorders that may arise in a patient in whom the nervous system has been damaged by, *e.g.*, trauma, surgery, stroke, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, or toxic agents; motoneuron disorders, such as amyotrophic lateral sclerosis (Lou Gehrig's disease), Bell's palsy, and various conditions involving spinal muscular atrophy, or paralysis; human "neurodegenerative disorders", such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, Huntington's chorea, Down's Syndrome, nerve deafness, and Meniere's disease; neuropathy, and especially peripheral, referring to a disorder affecting the peripheral nervous system, most often manifested as one or a combination of motor, sensory, sensorimotor, or autonomic neural dysfunction, such as distal sensorimotor neuropathy, or autonomic neuropathies including reduced motility of the gastrointestinal tract or atony of the urinary bladder. Examples of neuropathies associated with systemic disease include post-polio

syndrome; examples of hereditary neuropathies include Charcot-Marie-Tooth disease, Refsum's disease, Abetalipoproteinemia, Tangier disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, and Dejerine-Sottas syndrome; and examples of neuropathies caused by a toxic agent include those caused by treatment with a chemotherapeutic agent such as vincristine, cisplatin, methotrexate, or 3'-azido-3'-deoxythymidine. Also, NRG3 or biologically active fragments thereof (such as an EGF-like domain of an NRG3) may be used to treat diseases of skeletal muscle or smooth muscle, such as muscular dystrophy or diseases caused by skeletal or smooth muscle wasting.

Semipermeable, implantable membrane devices are useful as means for delivering drugs in certain circumstances. For example, cells that secrete soluble NRG3, or agonist thereof, or chimeras can be encapsulated, and such devices can be implanted into a patient, for example, into the brain of patients suffering from Parkinson's Disease. See, U.S. Patent No. 4,892,538 of Aebischer et al.; U.S. Patent No. 5,011,472 of Aebischer et al.; U.S. Patent No. 5,106,627 of Aebischer et al.; PCT Application WO 91/10425; PCT Application WO 91/10470; Winn et al. (1991) *Exper. Neurology* 113:322-329; Aebischer et al. (1991) *Exper. Neurology* 111:269-275; and Tresco et al. (1992) *ASAIO* 38:17-23. Accordingly, also included is a method for preventing or treating damage to a nerve or damage to other NRG3-expressing or NRG3-responsive cells, e.g. brain, heart, or kidney cells, as taught herein, which method comprises implanting cells that secrete NRG3, or fragment or agonist thereof, or antagonist as may be required for the particular condition, into the body of patients in need thereof. Finally, the present invention includes an implantation device, for preventing or treating nerve damage or damage to other cells as taught herein, containing a semipermeable membrane and a cell that secretes NRG3, or fragment or agonist thereof, (or antagonist as may be required for the particular condition) encapsulated within the membrane, the membrane being permeable to NRG3, or fragment agonist thereof, and impermeable to factors from the patient detrimental to the cells. The patient's own cells, transformed to produce NRG3 *ex vivo*, could be implanted directly into the patient, optionally without such encapsulation. The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished readily as is known in the art. The present

invention includes, therefore, a method for preventing or treating cell damage, preferably nerve damage, by implanting cells into the body of a patient in need thereof, the cells either selected for their natural ability to generate NRG3, or fragment or agonist thereof, or engineered to secrete NRG3, or fragment or agonist thereof. Preferably, the secreted NRG3 is soluble, human NRG3 when the patient is human. The implants are preferably non-immunogenic and/or prevent immunogenic implanted cells from being recognized by the immune system. For CNS delivery, a preferred location for the implant is the cerebral spinal fluid of the spinal cord.

The administration of the NRG3, fragment or variant thereof, of the present invention can be done in a variety of ways, e.g., those routes known for specific indications, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraarterially, intralesionally, intraventricularly in the brain, or intraocularly. The NRG3 may be administered continuously by infusion into the fluid reservoirs of the CNS, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. Sustained release systems can be used. Where the disorder permits, one may formulate and dose the NRG3 variant for site-specific delivery. Administration can be continuous or periodic. Administration can be accomplished by a constant- or programmable-flow implantable pump or by periodic injections.

Semipermeable, implantable membrane devices are useful as means for delivering drugs in certain circumstances. For example, cells that secrete soluble NGF variant can be encapsulated, and such devices can be implanted into a patient, for example, into the brain or spinal chord (CSF) of a patient suffering from Parkinson's Disease. See, U.S. Patent No. 4,892,538 of Aebischer et al.; U.S. Patent No. 5,011,472 of Aebischer et al.; U.S. Patent No. 5,106,627 of Aebischer et al.; PCT Application WO 91/10425; PCT Application WO 91/10470; Winn et al. (1991) *Exper. Neurology* 113:322-329; Aebischer et al. (1991) *Exper. Neurology* 111:269-275; and Tresco et al. (1992) *ASAIO* 38:17-23. Finally, the present invention includes an implantation device, for preventing or treating nerve damage or damage to other cells as taught herein, containing a semipermeable membrane and a cell that secretes an NRG3, the cell

being encapsulated within the membrane, and the membrane being permeable to NRG3, but impermeable to factors from the patient detrimental to the cells. The patient's own cells, transformed to produce NRG3 *ex vivo*, optionally could be implanted directly into the patient without such encapsulation. The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished readily as is known in the art. Preferably, the secreted NRG3, fragment or variant thereof, is a human NRG3 when the patient is human. The implants are preferably non-immunogenic and/or prevent immunogenic implanted cells from being recognized by the immune system. For CNS delivery, a preferred location for the implant is the cerebral spinal fluid of the spinal cord.

The pharmaceutical compositions of the present invention comprise a NRG3 in a form suitable for administration to a patient. In the preferred embodiment, the pharmaceutical compositions are in a water soluble form, and may include such physiologically acceptable materials as carriers, excipients, stabilizers, buffers, salts, antioxidants, hydrophilic polymers, amino acids, carbohydrates, ionic or nonionic surfactants, and polyethylene or propylene glycol. The NRG3 may be in a time-release form for implantation, or may be entrapped in microcapsules using techniques well known in the art.

An effective amount of NRG3 or NRG3 agonist or antagonist to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 10 ng/kg to up to 100 mg/kg of patient body weight or more per day, preferably about 1 µg/kg/day to 10 mg/kg/day. Typically, the clinician will administer NRG3 or NRG3 agonist or antagonist until a dosage is reached that achieves the desired effect for treatment of the above mentioned disorders.

L. Transgenic and Knockout Animals

Nucleic acids which encode novel NRG3 from non-human species, such as the murine NRG3, can be used to generate either transgenic animals or "knock out" animals which, in turn,

are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, murine cDNA encoding NRG3 or an appropriate sequence thereof can be used to clone genomic DNA encoding NRG3 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding NRG3. Methods for generating transgenic animals, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells, such as neuronal cells, would be targeted for NRG3 transgene incorporation with tissue-specific enhancers, which could result in altered cell differentiation, cell proliferation, or cellular apoptosis, depending upon the ligand interaction with the expressed polypeptide. Transgenic animals that include a copy of a transgene encoding NRG3 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding NRG3. Such animals can be used as tester animals for reagents thought to confer protection from, for example, diseases associated with abnormal neuronal differentiation and neuronal cell proliferation, for example. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the disease, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the disease.

Alternatively, the non-human homologues of NRG3 can be used to construct a NRG3 "knock out" animal which has a defective or altered gene encoding NRG3 as a result of homologous recombination between the endogenous gene encoding NRG3 and altered genomic DNA encoding NRG3 introduced into an embryonic cell of the animal. For example, murine cDNA encoding NRG3 can be used to clone genomic DNA encoding NRG3 in accordance with established techniques. A portion of the genomic DNA encoding NRG3 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas and Capecchi, *Cell* 51:503 (1987) for a

description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected (see, e.g., Li *et al.*, Cell 69: 915 (1992)). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see, e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be used in the selection of potential therapeutic agents, such as NRG3 agonists, that restore the cellular processes initiated or maintained by native NRG3; or the knockout animals can be used in the study of the effects of *nrg3* mutations.

The instant invention is shown and described herein in what is considered to be the most practical, and the preferred embodiments. It is recognized, however, that departures may be made therefrom which are within the scope of the invention, and that obvious modifications will occur to one skilled in the art upon reading this disclosure.

EXAMPLES

The following examples are provided so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make the compounds and compositions of the invention and how to practice the methods of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to insure accuracy with respect to numbers used (e.g. amounts, temperature, etc.), but some experimental errors and deviation should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in degrees C, and pressure is at or near atmospheric.

Example 1: Molecular cloning of a mouse and human novel NRG3

Novel NRG3 cDNAs were identified using an expressed sequence tag shown below:

AATTTCTGCCGAAAAGTATTCCATCTTATCGGATCCAACAGACCACTTGGGGATT
GAATTCATGGAGAGTGAAGAAGTTTATCAAAGGCAGGTGCTGTCAATTTTCATGTAT
CATCTTTGGAATTGTCATCGTGGGCATGTTCTGTGCAGCATTCTACTTCAAAAGCAA
GAAACAAGCTAAACAAATCCAAGAGCAGCTGAAAGTGCCACAAAATGGTAAAAGC
TACAGTCTCAAAGCATCCAGCACAATGGCAAAGTCAGAGAACTTGGTGAAGAGCCA
TGTCAGCTGCAAAAATAAAATGTCAGGCTTCTGAGCCCAAGCTAAGCCATCATATC
CCCTGTGACCTGCACGTGCACATCCXGATGGCCCGTTTCCTGCCTTTTGTGATGAC
ATTTXCACCACAAATGXAGTGAAAATGGGXCTTTTCXTGCCTTAAGTTGACXT
TTTTXCCCCAAAAGGAG (EST; SEQ ID NO:21; Genbank entry H23651) from the National
Center for Biotechnology Information (NCBI) database of ESTs. This EST from a human brain
cDNA library, encodes an amino acid sequence having approximately 62% identity to amino
acids 232-316 of heregulin- β 1 (also designated neuregulin- β 1, or NRG1).

To obtain a partial human cDNA clone, a 50-base single stranded oligonucleotide probe
(5'-TGGTAAAAGCTACAGTCTCAAAGCATCCAGCACAATGGCAAAGTCAGAGA-3';
SEQ ID NO:18) was synthesized based on the EST sequence. The probe was used to screen 1.5×10^6
plaques from a λ gt10 cDNA library prepared from human fetal brain RNA (HL3003a,
Clontech) as described by Godowski et al. (Godowski, P.J. et al. (1989) PNAS USA 86:8083-
8087, herein incorporated by reference in its entirety). Nine positive plaques were obtained and
the sequences of both strands of the largest inserts were determined by standard sequencing
techniques. From these cloned overlapping sequences, a partial cDNA sequence of the human
NRG3 was obtained.

Additional 5' human NRG3 sequence was obtained by anchored PCR of human
hippocampus RNA (Clontech). The complete human open reading frame nucleic acid sequence
deduced from direct sequencing of hNRG3B1 cDNA is shown in Fig. 2 (SEQ ID NO:5). ATCC
_____ is nucleic acid comprising an expression vector and the nucleotide sequence of the
human NRG3B1 open reading frame. An alternatively spliced form of human NRG3 was cloned
as pRK5.tk.neo.hNRG3B2 (SEQ ID NO:22) encoding the deduced amino acid sequence of SEQ

ID NO:23, which amino acid sequence lacks amino acids 529 to 552 of SEQ ID NO:6 (see Fig. 4B). Since this alternatively spliced form of human NRG3 comprises the EGF-like domain of the other NRG3s as well as high amino acid sequence homology, it is expected to exhibit the biological properties of the NRG3s disclosed herein.

To clone murine NRG3 cDNA sequences, two degenerate primers were designed based on regions proximal to the transmembrane domain of the partial human cDNA, encoding the amino acid sequences NDGE CFVI (SEQ ID NO:19) and EFMESEEVY (SEQ ID NO:20). A mouse brain cDNA library (Clontech, ML1042a) was screened, and a clone (C5a) containing a partial murine NRG3 cDNA was obtained by standard techniques. Using a probe derived from the C5a sequence, two additional mouse brain cDNA libraries (ML1034h, Clontech; and 936309, Stratagene) were screened. Both strands of two overlapping murine partial NRG3 clones, SWAJ-3 and ZAP-1 were sequenced and, together were found to encode an entire open reading frame (ORF) of 2139 bp having the DNA sequence SEQ ID NO:1 and the deduced amino acid sequence SEQ ID NO:2 shown in Fig. 4A. Nucleic acid comprising the murine NRG3 open reading frame cloned into an expression vector is designated pLXSN.mNRG3 (ATCC _____).

The chromosomal localization of human NRG3 was mapped to 10q22 by PCR analysis of somatic cell hybrid DNA, whereas the NRG1 gene is located at 8p11-22 (Lee, J. and Wood, W. I. (1993) Genomics 16 :790-791; and Orr-Urtreger, A. et al.. (1993) Proc. Natl. Acad. Sci. USA 90:1867-1871). Thus, NRG3 is a novel member of the EGF-like family of protein ligands.

Example 2:.. Characterization of the Mouse and Human NRG3 Deduced Amino Acid Sequences.

The cDNAs of human and murine NRG3 contained open reading frames encoding proteins of 720 and 713 amino acids respectively, with predicted MW of 77,901 Da for human NRG3 and 77,370 Da for murine NRG3 (Fig. 4). The two species of NRG3 are 93% identical in amino acid sequence.

Analysis of the amino acid sequence of human NRG3 revealed that it contained homology to NRG1 family members (i.e. 23% and 19% sequence identity to SMDF (Ho, W. H. et al. (1995) J. Biol. Chem. 270:14523-32) and heregulin- β 1 (Holmes, W. E. et al. (1992) Science 256:1205-10) respectively). A hydropathy analysis indicated two hydrophobic segments:

W⁶⁶-V⁹¹ and L³⁶²-F³⁸³ (amino acid numbers according to human NRG3). Similar to NRG1, the C-terminal hydrophobic segment may serve as the transmembrane domain and the N-terminal region may act as internal signal sequence (Wickner, W. T. and Lodish, H. F. (1985) *Science* **230**:400-7; Sabatini, D. D. et al. (1982) *J. Cell Biol.* **92**:1-22; and Blobel, G. (1980) *Proc. Natl. Acad. Sci. USA* **77**:1496-500). In contrast to many neuregulin family members, the extracellular domain of NRG3 is devoid of Ig-like or kringle domains. Instead, NRG3 contains a unique Ala/Gly rich segment at the N-terminus, a mucin-like Ser/Thr rich region containing abundant sites for O-linked glycosylation, and an EGF motif. There are no predicted sites for N-linked glycosylation. The EGF-like domain of NRG3 is distinct from those encoded by the NRG1 (31% identity compared with neuregulin- β 1 EGF-like domain) and NRG2 (39% identity with neuregulin- β 1 EGF-like domain), suggesting that NRG3 is not an alternatively spliced NRG1 isoform. A diagrammatic comparison of EGF-like domains of EGF family members is shown in Fig. 5. The putative intracellular domain of NRG3 contains only approximately 13% sequence identity to the intracellular domain of NRG1. The EGF-like domains of the EGF family members were obtained from the following sources, each reference herein incorporated by reference in its entirety. The sequences compared in Fig. 5 include the EGF-like domain of human NRG3 (hNRG3.egf; SEQ ID NO:4; disclosed herein); chicken ARIA (cARIA.egf; SEQ ID NO:9) (Falls, D.L. et al. (1993) *Cell* **72**:801-815), human amphiregulin (hAR.egf; SEQ ID NO:10) (Plowman, G. D. et al. (1990) *Mol. Cell. Biol.* **10**:1969-81.); human betacellulin (hBTC.egf; SEQ ID NO:11) (Sasada, R. et al. (1993) *Biochem. Biophys. Res. Com.* **190**:1173-9); human EGF (hEGF.egf; SEQ ID NO:12) (Nagai, M. et al. (1985) *Gene* **36**:183-8.); human heparin-binding EGF-like growth factor (hHB-EGF.egf; SEQ ID NO:13) (Higashiyama, S. et al. (1991) *Science* **251**:936-9.); human heregulin- α (hHRG α ; SEQ ID NO:14); human heregulin- β (hHRG β .egf; SEQ ID NO:15) (Holmes, W.E. et al. (1992) *Science* **256**:1205-1210); human TGF- α (hTGF α .egf; SEQ ID NO:16) (Derynck, R. et al. (1984) *Cell* **38**:287-97.); and mouse epiregulin (mEPR.egf; SEQ ID NO:17) (Toyoda, H. et al. (1995) *FEBS Lett.* **377**:403-7.).

Example 3: Expression of murine and human NRG3

A. Northern Blot Analysis of Human tissue. The tissue expression profile of the human

NRG3 was examined by Northern blot analysis. A multi-tissue RNA blot containing 2 µg each of poly(A)⁺ RNA from human tissues were purchased from Clontech. The region of the human NRG3 nucleic acid sequence encoding amino acids 394 to 536 was used to generate DNA hybridization probes by PCR amplification. The DNA probes were labeled with α-³²P-dCTP by random priming (Promega). The RNA blot was hybridized with 50% formamide, 5 x SSC, 50 mM potassium phosphate (pH 7.0), 5 x Denhardt's, 10% dextran sulfate at 42 °C for 20 hr. The blot was washed with 0.1 x SSC, 0.1%SDS at 50 °C for 30 min and exposed in PhosphoImager™. Expression of NRG3 in mixtures of tissues was used as a guide to determine expression in specific tissues by *in situ* hybridization.

B. *In situ* Hybridization Analysis of Mouse Tissues. Formalin-fixed, paraffin-embedded mouse embryos (embryonic days 13, 14, 16), and glutaraldehyde-fixed, paraffin-embedded or paraformaldehyde-fixed, frozen adult mouse brain, ovary, jejunum, kidney, adrenal, lung, stomach, spleen, skeletal muscle, liver and colon were sectioned and processed for *in situ* hybridization by the method of Lu and Gillett (Lu, L.H. and Gillett, N.A. (1994) Cell Vision 1:169-176) with modifications. Briefly, the *in situ* hybridization probe was generated by *in vitro* transcription directly from a PCR fragment, rather than from a plasmid DNA as described. ³²P-UTP-labeled sense and antisense riboprobes were generated by labeling PCR products of a cDNA fragment encoding amino acids C²⁹² to N⁴⁸² of murine NRG3.

C. Northern Blot And *In Situ* Hybridization Analyses Reveal a Neural Expression Pattern of NRG3. A 4.4 kb mRNA transcript that hybridized to the probe derived from amino acids 394 to 536 of human NRG3 was highly expressed in brain. In a Northern blot of various brain tissues, NRG3 expression was detected at high levels in most regions of the brain with the exception of corpus callosum. A lower level expression of a 1.9-kb transcript was detected in testis. The 4.4-kb transcript, but not the 1.9-kb transcript, is of sufficient size to encode NRG3, suggesting that the smaller transcript may encode an alternatively spliced form of NRG3. A similar pattern of expression of NRG3 was observed in RNA blots from murine tissues using a probe derived from the region of murine NRG3 that overlaps the EGF-like domain.

The tissue distribution of NRG3 expression was characterized by *in situ* hybridization using tissues of embryonic and adult mice. At embryonic day 13 (E13) (the earliest time point examined), NRG3 mRNA was confined to the nervous system. A strong signal for NRG3 mRNA in the brain, spinal cord, trigeminal, vestibular-cochlear and spinal ganglia of embryonic day 16 (E16) mice was also demonstrated. Regions of the telencephalon containing differentiating cells (e.g., the cortical plate) displayed an intense NRG3 signal, whereas the underlying regions containing proliferating or migrating cells (ventricular and subventricular zones), showed little expression. Thus, NRG3 appeared to be expressed mainly in the nervous system of embryonic mice. In adult animals NRG3 antisense probes hybridized to mRNA in spinal cord and numerous brain regions including deep cerebellar nuclei, vestibular nuclei, cerebral cortex, piriform cortex, anterior olfactory nucleus, medial habenula, hippocampus, hypothalamus and thalamus.

Example 4: Characterization of the binding characteristics of NRG3 fragments

A. Expression and Purification of NRG3^{EGF} Fusion Protein in Mammalian Cells

To examine the binding characteristics of the NRG3 EGF-like domain as well as to demonstrate the functionality of an NRG3 fragment of the invention, a soluble fusion protein was prepared comprising a sequence of EGF-like domain, which domain has the same amino acid sequence in mouse and human NRG3.

A secreted, epitope tagged polypeptide comprising the EGF-like domain of murine NRG3₂₈₄₋₃₄₄ was constructed by linking in the expressed N-terminal to C-terminal direction 1) the coding sequence for the gD signal sequence and epitope tag (Mark, M. R. et al. (1994) *J. Biol. Chem.* 269, 10720-10728); 2) the sequences encoding amino acids 284-344 of murine NRG3 (identical to human NRG3 amino acids 282 to 342); and 3) the coding sequences of the Fc portion of human IgG₁ in pSAR.SD5 vector (psar.SD5, from A. Shen, Genentech, Inc.). The plasmid encoding these sequences was designated NRG3^{EGF}.Fc. The NRG3^{EGF}.Fc expression plasmid was transfected using LipofectAMINE (GIBCO/BRL, Bethesda, MD) into DHFR⁻ Chinese hamster ovary cells (CHO/DP12; ATCC designation CCL 9096). Clones were selected in glycine/hypoxanthine/thymidine minus medium see, for example, (Sambrook, J. et al.,

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1989)), pooled, and expanded. The encoded fusion protein was expressed in cultures of the selected clones. Conditioned media from these cells were collected and the recombinant protein purified by a HiTrap protein A affinity column (Pharmacia).

5 A monomeric fusion protein designated NRG3^{EGF}.H6 fusion protein was produced in the same system as the Fc-fusion protein and purified through a cobalt affinity column. NRG3^{EGF}.H6 comprises the N-terminal gD tag, murine NRG3₂₈₄₋₃₄₄, and a coding sequence for six histidine residues. Purification was based on the affinity of the histidine side chains for immobilized cobalt using a cobalt affinity column (Cobalt affinity column, R. Vandlen, Genentech, Inc.).
10 Protein concentration was determined by BioRad Protein Assay (BioRad, Richmond, CA).

B. Generation of K562^{erbB} Cell Lines. Stable cell lines that expressed human ErbB2, ErbB3 or ErbB4 receptors were derived from K562 cells (K562 cells have ATCC designation CCL 243). cDNAs of human *erbB2*, *erbB3* and *erbB4* were from L. Bald and G. Scoffer, Genentech (Sliwkowski, M. et al. (1994), J. Biol Chem. 269:14661-14665). These cDNAs were subcloned into CMV-based expression vectors and introduced into the K562 human myeloid leukemia cell line by electroporation (1180 mF, 350 V). The transfectants were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10 mM HEPES containing 0.8 mg/ml G418. Resistant clones were
15 obtained by limiting dilution, and receptor expression was confirmed by western blot and NRG binding assays. Receptor expression was confirmed by western blot analysis using antibodies for each of the ErbB receptors (antibodies prepared at Genentech, Inc.) Phorbol ester stimulation was found to significantly enhance receptor expression in both the ErbB3 and ErbB4 transfectants, and the stably transfected K562 cell lines were cultured in medium containing 10
20 ng/ml Phorbol, 12-Myristate, 12-Acetate- (PMA) overnight prior to use.

C. FACS Analysis. For each binding reaction, 5×10^5 stably transfected K562 cells were suspended in PBS/2%BSA at 4 °C for 30 min followed by incubation with 5 µg of isolated, purified NRG3^{EGF}.Fc (MW 90 kDa) in a volume of 0.25 ml on ice for 60 min. 1 µg of primary
25

antibody (anti-gD or anti-ErbB receptor) and secondary PE-conjugated (CALTAG, CA., goat anti-mouse, 1:100 dilution) antibodies were added sequentially with 30-60 min incubation time and extensive washes before each addition. FACS analyses were performed on a Becton & Dickson FACS instrument. Anti-gD (5B6), anti-ErbB2 receptor (4D5), anti-ErbB3 receptor (2F9) and anti-ErbB4 receptor (3B9) monoclonal antibodies were prepared using standard techniques by the Monoclonal Antibody Group, Genentech, Inc.

D. The EGF-Like domain of NRG3 Binds Specifically to the ErbB4 Receptor Tyrosine Kinase. To identify the receptor(s) for NRG3, the ability of NRG3 to bind to any of the known neuregulin receptors was investigated. Stable cell lines were generated which expressed receptors ErbB2, ErbB3, or ErbB4. The parental cell line K562 does not express detectable levels of ErbB receptors (Fig. 6A). K562^{erb2}, K562^{erb3} and K562^{erb4} cells expressed only the corresponding receptors (Figs. 6B-6D).

Since the EGF-like domain determines the binding specificity of NRG1 to their receptors, a protein containing an epitope tagged version of the EGF-like domain of NRG3 fused to the Fc portion of human IgG was expressed and purified. Using a FACS assay, it was observed that NRG3^{EGF}.Fc bound to cells expressing ErbB4 receptor (Fig. 6H). Binding was specific in that NRG3^{EGF}.Fc did not bind to either the parental K562 cells, or cells expressing either ErbB2 or ErbB3 (Fig. 6E-6G). A control fusion protein, RSE.Fc, did not bind to any of these cell lines. This binding of NRG3^{EGF}.Fc to K562^{erb4} cells was competed in a dose-dependent fashion by the EGF-like domain of heregulin- β 1 (NRG1^{EGF}), but not by RSE.Fc, suggesting that NRG3^{EGF}.Fc interacts directly with ErbB4 receptors on the cell surface.

A soluble form of the ErbB4 receptor was co-immunoprecipitated by NRG3^{EGF}.Fc *in vitro*, further demonstrating the binding of NRG3^{EGF}.Fc to ErbB4 receptor.

The binding of NRG3^{EGF}.Fc to ErbB4 receptor was further analyzed by direct competitive binding assays using ¹²⁵I-labeled NRG3^{EGF}.Fc. Purified NRG3^{EGF}.Fc was radio-iodinated using the lactoperoxidase method as described by Sliwkowski et al. (Sliwkowski, M. X. et al., (1994) *J. Biol. Chem.* **269**, 14661-5). The average specific activity of the radiolabeled protein was 300 μ Ci/ μ g. Binding of ¹²⁵I-NRG3^{EGF}.Fc to immobilized ErbB4.Fc was competed by either

NRG3^{EGF}.Fc or EGF domain of NRG1^{EGF} (rHRGβ1₁₇₇₋₂₄₄) in a concentration dependent manner.

The displacement binding assays were performed in Maxisorp C 96-wells (Nunc, Naperville, Illinois). Goat anti-human antibody (Boehringer Mannheim, Germany) was coated on the plate at a concentration of 0.2 µg/well in 100 µl of 50 mM sodium carbonate buffer (pH 9.6) at 4 °C, overnight. The plate was blocked by 1%BSA in TBST buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.02% Tween 20) for 30 min at room temperature (RT). A soluble form of ErbB4 receptor was added at 15 ng/well in 1%BSA/TBST and incubated for 1.5 hr at RT. To prevent radiolabeled protein from interacting with residual goat anti-human antibodies, 1 µM of a humanized monoclonal antibody (rhuMAB HER2; Carter, P. et al. (1992) Proc. Natl. Acad. Sci. USA 89:4285-9) was added to the plate for 20 min and was included in the subsequent binding reaction.

The competitive binding assay was then initiated by the addition of 80 pM (200,000 cpm) of ¹²⁵I-NRG3^{EGF}.Fc along with various concentrations of unlabeled NRG3^{EGF}.Fc or NRG1^{EGF} (*E. coli*-expressed, without Fc). NRG1^{EGF} is the EGF domain of NRG1, corresponding to amino acids 177-244 of the neuregulin-β1 isoform (Holmes, W. E. et al. (1992) Science 256:1205-10) and obtained from J. A. Lofgren, Genentech, Inc. The final incubation volume was 100 µl in binding buffer (F-12/DMEM medium, 50 mM HEPES, pH7.5, 2%BSA) and the reaction was allowed to proceed at RT for 1.5 hr. The unbound material was washed by TBST extensively, and the bound radioactivity was counted on a Beckman IsoData gamma-counter (Smith-Kline Beckman, PA). Data was analyzed using a nonlinear regression computer program.

Based on the results of the binding experiments as shown in Figs. 6A-6H, the estimated affinity (K_d) for NRG3^{EGF}.Fc for binding to ErbB4.Fc was determined to be 9 ± 4 nM (n = 4), and the apparent K_i of NRG1^{EGF} was approximately 1 nM. The shallowness of the displacement curve of NRG3^{EGF}.Fc may be due to the fact that the NRG3^{EGF}.Fc is expressed as a bivalent Fc fusion protein (Fig. 7). The results of the control experiments showed that ¹²⁵I-NRG3^{EGF}.Fc did not bind control receptor RSE.Fc in the same experiment, and RSE.Fc did not compete ¹²⁵I-NRG3^{EGF}.Fc bound to ErbB4.Fc.

E. Tyrosine Phosphorylation Assay. NRG1 binds and activates ErbB2, ErbB3 and

ErbB4 receptor resulting in tyrosine phosphorylation and downstream signaling events (Sliwkowski, M.X., et al. (1994), *supra*; Plowman, G. D. et al. (1993) *supra*; and Carraway, K.L. and Cantley, L.C. (1994), *surpa*). As demonstrated in the preceding example, NRG3 binds ErbB4 receptor, but not ErbB2 or ErbB3 receptors at a detectable level. The ability of the EGF-like domain of NRG3 (NRG3^{EGF}) to activate ErbB4 receptor, K562^{erbB4} cells was examined.

K562^{erbB4} cells or MDA-MB-453 cells (negative control; ATCC designation HB 131) were cultured in medium lacking serum for 12 hours and then stimulated with NRG3^{EGF}.Fc, NRG^{EGF}.H6 or NRG1^{EGF}. K562^{erbB4} cells were treated with 2.5 nM or 25 nM of NRG3^{EGF}.Fc for 3 min or 8 min. As a positive control, the cells were similarly treated with NRG1^{EGF}.

ErbB4 receptor tyrosine phosphorylation was detected by immunoprecipitation and Western blot according to the following procedure. Cells were lysed with lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 30 mM NaF, 2 mM EDTA, 2 mM EGTA, 0.1% SDS, 1% Triton X-100, 2 mM sodium vanadate, 2 mM sodium molybdate, 2 mM of PMSF). After removing cell debris by centrifugation, 1 µg of anti-ErbB4 receptor monoclonal antibody (C-18, Santa Cruz Biotechnology, Santa Cruz, CA) was added together with 20 µl of protein A-agarose slurry (Sigma, St. Louis, MO). Immunoprecipitation was performed at 4 °C overnight, complexes were collected by centrifugation and washed three times with 1 ml lysis buffer. Proteins were separated by reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on Novex 4%-12% minigels and transferred to nitrocellulose. The blots were probed with peroxidase conjugated anti-phosphotyrosine antibody (Transduction Laboratory). The blot was stripped and reprobed with anti-ErbB4 receptor antibody followed by peroxidase conjugated goat anti-rabbit IgG antibody (Sigma) to visualize ErbB4 receptor proteins.

Based on these experiments, it was demonstrated that NRG3^{EGF}.Fc stimulated ErbB4 receptor tyrosine phosphorylation at both time points and in a dose dependent manner.

To confirm the ability of NRG3^{EGF} to activate the ErbB4 receptor tyrosine phosphorylation, receptor activation in the human breast cancer cell line MDA-MB-453 was examined. This cell line expresses high level of ErbB2 and ErbB3 receptors, and low levels of ErbB4 receptor. Treatment of MDA-MB-453 cells with NRG3^{EGF}.Fc or with a monomeric form

of the EGF domain (NRG3^{EGF}.H6) resulted in substantial increase of tyrosine phosphorylation of ErbB4 receptor.

NRG family members and other members in the EGF family display a complex pattern of receptor binding. In most cases, one ligand is able to bind several combinations of receptor homo- and heterodimers (Karunakaran, D. et al. (1996) *EMBO J* **15**:254-264, Beerli, R. R. and Hynes, N. E. (1996) *J. Biol. Chem.* **271**:6071-6076). For example, NRGs bind ErbB2/ErbB3 receptor heterodimers and ErbB4/ErbB4 receptor homodimers with high affinity but ErbB3/ErbB3 receptor homodimers with low affinity (Sliwkowski, M. X. et al.. (1994) *J. Biol. Chem.* **269**, 14661-5, Carraway, K. L. and Cantley, L. C. (1994) *Cell* **78**, 5-8, Tzahar, E. et al. (1994) *J. Biol. Chem.* **269**, 25226-33, Carraway, K. L. et al. (1994) *J. Biol. Chem.* **269**, 14303-6, and Kita, Y. A. et al. (1994) *FEBS Lett.* **349**, 139-43). Betacellulin binds both EGFR and ErbB4 homodimers (Riese, D. J. et al. (1995) *Mol. Cell. Biol.* **15**:5770-6). The EGF-like domains of EGF and NRG1 family members determine the specificity of receptor activation (Barbacci, E. G. et al. (1995) *J. Biol. Chem.* **270**:9585-9589). The limited amino acid sequence homology in the EGF-like domains of NRG3 and NRG1 suggests that NRG3 may have a different spectrum of receptor interactions relative to members of the NRG family, but with potentially overlapping binding sites, since binding of the EGF-like domain of NRG3 to ErbB4 can be competed by the EGF-like domain of NRG1.

NRG3^{EGF}.Fc did not bind to K562 cells that express either ErbB2 or ErbB3 (Fig. 6E-6G), or to MDA-MB-486 cells which express high levels of the EGFR. An increase in phosphorylation of either the EGFR, ErbB2 or ErbB3 in MDA-MB-453 cells treated with NRG3 also was not observed.

Most variants of NRGs, with the exception of the neural specific form of SMDF, are widely expressed in numerous tissues including brain, heart, skeletal muscle, breast, liver, lung, among others. Betacellulin, a ligand for both EGFR and ErbB4, also displays broad tissue expression patterns (Shing, Y. et al. (1993) *Science* **259**, 1604-7; Sasada, R. et al. (1993) *Biochem. Biophys. Res. Com.* **190**, 1173-9). In contrast, the expression of NRG3 is strikingly restricted to neural tissues as disclosed herein by Northern analysis and *in situ* hybridization. Developmentally, NRG3 mRNA can be detected as early as E11 (but not E4) in mouse as judged

by Northern blot and E13 by *in situ* hybridization (the earliest age examined). ErbB4 is predominantly expressed in brain, heart and skeletal muscle (Plowman, G. D. et al. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1746-50). ErbB4 was also shown to be broadly distributed in the brains of chick embryos (E14, E17, predominantly in neurons) (Francoeur, J. R. et al. (1995) *J. Neur. Res.* **41**, 836-45), in rat retina cultures (Bermingham-McDonogh, O. et al. (1996) *Development* **122**, 1427-38.), at neuromuscular synapses (Zhu, X. et al. (1995) *EMBO J.* **14**, 5842-8.), but not in cultured human and rat Schwann cells (Grinspan, J. B. et al. (1996) *J. Neuroscience* **16**, 6107-6118, Levi, A. D. et al. (1995) *J. Neuroscience* **15**, 1329-40.). Recently, ErbB4 was found to co-localize with GABA⁺ cells (Weber, J. et al. (1996) *Soc Neurosci Abstr* **22**, 1579.). Thus, the same receptor may mediate distinct biological functions in different tissues or cell types when interacted with corresponding tissue-specific ligands. For example, NRG1 may serve as a ligand for ErbB4 during heart development, betacellulin may act as a mitogenic ligand for ErbB4 in variety of cell types, while neural specific ligand(s) (such as NRG3) may function as trophic or guidance molecules on ErbB4 receptor expressing cells in the central or peripheral nervous systems.

Example 5: Binding and ErbB4 receptor tyrosine kinase activation by full length mouse and human NRG3s.

A full length murine NRG3 or human NRG3 was synthesized based on the murine and human consensus nucleic acid sequences SEQ ID NO:1 and SEQ ID NO:5, respectively and the NRG3s were expressed as amino acid sequences. Based on the experiments described herein for the characterization of NRG3-EGF binding and ErbB4 receptor activation, analogous experiments are performed for the full length consensus NRG3 from mouse and human sources. Adjustments to the reaction conditions are made to optimize pH, solutes and their concentrations, and other relevant parameters to allow ErbB4 receptor-binding of the full length consensus NRG3 and ErbB4 receptor activation.

Alternatively, a murine or human NRG3 polypeptide fragment comprising the EGF-like domain but lacking the transmembrane domain is synthesized and tested for ErbB4 receptor binding and activation as described herein. Such a NRG3 fragment may, for example, include

the extracellular domain of a NRG3, which extracellular domain contains the EGF-like domain.

A NRG3 extracellular domain may optionally be fused to an immunoglobulin constant region, as shown herein for the NRG3-EGF-Fc fusion proteins. As an Fc fusion protein, the NRG3 extracellular domain-Fc protein is expected to form a dimer. The immunoglobulin constant region is preferably from IgG, but may also be taken from IgM, IgA and IgE and remain within the scope of the invention.

Where a monomeric fusion protein is desired that retains binding activity or binding and activation ability, the extracellular domain is fused to, for example, a series of histidine residues as disclosed herein for the NRG3-EGF-H6 immunoadhesion.

Adjustments to the binding reaction conditions are made to optimize pH, solutes and their concentrations and other relevant parameters to allow ErbB4 receptor-binding of the NRG3 fragment and ErbB4 receptor activation.

Example 6: Enhancement of cellular proliferation

Enhancement of cellular proliferation is exemplified by the following assay in which cells expressing ErbB4 receptor on their surface are treated with NRG3. It is understood that according to the invention, the cells may be treated with a NRG3 fragment (such as the NRG3 EGF-like domain) or a NRG3 variant.

As an example, rat retina cells which naturally express ErbB4 receptor (Bermingham-McDonogh, O. et al. (1996) *Development* 122, 1427-38) are cultured by standard techniques. The cultured cells are contacted with NRG3 in a dose dependent manner and an increase in cell number (e.g. a 30% percent increase at 48 hours) and EC50 is determined.

NRG3 treatment may also alter the morphology of these cells; untreated cells were multipolar with numerous branched processes whereas NRG3-treated cells may become spindle-shaped smooth processes and/or align themselves in a parallel array.

NRG3 is believed to stimulate neuronal cell growth in a dose dependent manner. NRG3 alone is expected to produce a significant increase in neuronal cell number compared to control medium. A synergistic effect may be observed between other neuronal proliferation enhancers such as gas6 (growth arrest-specific gene; see, for example, Schneider *et al.*, Cell 54:787-793

(1988); and Manfioletti *et al.* in *Molec. Cell Biol.* 13(8):4976-4985 (1993)) and/or heregulin.

NRG3 is expected to increase both cell number and thymidine incorporation as measures of cell proliferation.

NRG3 is expected to have an effect on cell morphology as determined by viewing phase contrast micrographs of ErbB4 receptor-expressing neuronal cells grown in various media containing NRG3 alone or NRG3 plus other cell proliferation enhancing compounds such as heregulin, gas6, fetal bovine serum, and the like. Photomicrographs are taken after 96 hours of culture. The cells grown in the presence of NRG3 are expected to have processes which are not observed in cells grown in the absence of NRG3.

Cells are stained by immunofluorescence for markers specific for the cultured neuronal cells. Briefly, passaged ErbB4 receptor-expressing neuronal cells are contacted with NRG3 and cultured for 24 hours on laminin coated Chamber slides and fixed in 10% formalin in PBS. Fixed cells are blocked with 10% goat serum and incubated with rabbit derived anti-marker antibody at dilutions recommended by the distributor. Specific binding of the primary antibody is observed by staining with goat anti-rabbit IgG (Fab')₂-FITC conjugates. Cells are counter-stained with DNA dye propidium iodide. Negative controls are run on WI-38 cells which stain negative. Cells grown under these conditions are expected to show 100% immunofluorescent staining for the cell markers.

The ability of NRG3 to stimulate proliferation in ErbB4 receptor-expressing neuronal cells through the ErbB4 tyrosine kinase receptors may be investigated as follows. Cells are stimulated with various amounts of NRG3 (for example, 0 to 200 nM) for 15 min in a 37°C incubator. Cell lysates are prepared and immunoprecipitated with an anti-ErbB4 receptor antibody. Tyrosine phosphorylation of ErbB4 receptor is detected with 4G10 anti-phosphorylation antibody. Approximately 10⁶ cells are grown to near confluence in defined media. Cells are treated with NRG3 for 15 min in a 37°C incubator and lysed on ice with 1 ml of lysis buffer (20 mM HEPES, pH7.4, 135 mM NaCl, 50 mM NaF, 1 mM sodium vanadate and 1 mM sodium molybdate, 2 mM EDTA and 2 mM EGTA, 10% glycerol, 1%NP40, 1µM okadaic acid, 1 mM PMSF and 1 mM AEBSF). Cell lysates are clarified by centrifuging at 14000 x g at 4°C for 10 min. Immunoprecipitations are performed using approximately 1 µg of

rabbit anti-ErbB4 receptor antibody or 2 µl of rabbit anti-ErbB4 receptor antiserum. Immunocomplexes are collected with 10 µl of Protein A Sepharose CL-4B beads. Proteins are separated on Novex 4-12% gradient gel and transferred onto nitrocellulose membrane. Anti-phosphotyrosine immunoblots are performed using 4G10 mouse anti-phosphotyrosine antibody (UBI), goat anti-mouse horseradish peroxidase conjugate and ECL developing kit (Amersham). Addition of NRG3 to ErbB4 receptor-expressing neuronal cells is expected to cause autophosphorlation of ErbB4 receptor tyrosine residue(s).

It is beneficial to have populations of mammalian neuronal cells (preferably human cells) for use as cellular prostheses for transplantation into areas of damaged spinal cord in an effort to influence regeneration of interrupted central axons, for assisting in the repair of peripheral nerve injuries and as alternatives to multiple autografts. See Levi *et al.*, *J. Neuroscience* 14(3):1309-1319 (1994). The use of cell culture techniques to obtain an abundant source of autologous graft material from a small biopsy has already met with clinical success in providing human epidermal cells to cover extensive burns (Gallico et al., *N. Eng J. Med.* 311:338-451 (1984)). Accordingly, it is expected that the above approach will meet with success in mammals, including humans.

All documents cited throughout the specification as well as the references cited therein are hereby expressly incorporated by reference in their entirety. While the present invention is illustrated with reference to specific embodiments, the invention is not so limited. It will be understood that further modifications and variations are possible without diverting from the overall concept of the invention. All such modifications are intended to be within the scope of the present invention.

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
mouse NRG3 pLXSN.mNRG3	_____	_____
human NRG3B1 pRK5.tk.neo.hNRG3B1	_____	_____
human NRG3B2 pRK5.tk.neo.hNRG3B2	_____	_____

These deposits are made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

What is claimed is:

CLAIMS

1. A polypeptide comprising an amino acid sequence encoding an EGF-like domain, wherein the amino acid sequence has the binding characteristics of NRG3.
2. The polypeptide of claim 1 wherein the binding characteristics of NRG3 comprise
 - (a) binding to ErbB4 receptor but not to ErbB2 receptor or ErbB3 receptor under experimentally comparable conditions; and
 - (b) activation of ErbB4 receptor tyrosine phosphorylation.
3. The polypeptide of claim 1 wherein the amino acid sequence has at least 75% amino acid sequence homology to the amino acid sequence SEQ ID NO:4.
4. The polypeptide of claim 1, wherein the polypeptide binds to the ErbB4 receptor and stimulates tyrosine phosphorylation of the ErbB4 receptor.
5. A polypeptide that binds ErbB4 receptor, which polypeptide is selected from the group consisting of
 - (a) a polypeptide comprising an amino acid sequence having at least 75% sequence homology to the extracellular domain NRG3 (SEQ ID NO:3 or 7).
 - (b) a polypeptide comprising an amino acid sequence having at least 75% sequence homology to SEQ ID NO:2 or SEQ ID NO:6;
 - (c) a further mammalian homologue of polypeptide (a) or (b);
 - (d) a soluble form of any of the polypeptides (a) - (c) having a transmembrane domain that cannot anchor the polypeptide in a cell membrane; and
 - (e) a derivative of any of the polypeptides (a) - (d) having the binding characteristics of NRG3.

6. The polypeptide of claim 1 encoded by a NRG3 nucleic acid open reading frame sequence in ATCC deposit _____ (pLXSN.mNRG3).

7. The polypeptide of claim 1 encoded by a NRG3 nucleic acid open reading frame sequence in ATCC deposit _____ (pRK5.tk.neo.hNRG3B1).

8. The polypeptide of claim 1 encoded by a NRG3 nucleic acid open reading frame sequence in ATCC deposit _____ (pRK5.tk.neo.hNRG3B2).

9. The polypeptide of claim 1 which is devoid of a cytoplasmic domain, or devoid of a transmembrane domain that can anchor the polypeptide in a cell membrane, or both.

10. The polypeptide of claim 1 unaccompanied by native glycosylation.

11. The polypeptide of claim 1 which has a variant glycosylation.

12. An antagonist of the polypeptide of claim 1.

13. An agonist of the polypeptide of claim 1.

14. An isolated nucleic acid molecule encoding the polypeptide of claim 1.

15. The nucleic acid molecule of claim 14 further encoding the extracellular domain of a mammalian NRG3.

16. The nucleic acid molecule of claim 15, wherein the encoded extracellular domain has at least 75% amino acid sequence identity to the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:7.

17. The nucleic acid molecule of claim 14 wherein the encoded amino acid sequence is devoid of a cytoplasmic domain or a transmembrane domain that can anchor the polypeptide in a cell membrane, or both.

18. An expression vector comprising the nucleic acid molecule of claim 14 operably linked to control sequences recognized by a host cell transformed with the vector.

19. An expression vector according to claim 18 obtainable as ATCC _____ (pLXSN.mNRG).

20. An expression vector according to claim 18 obtainable as ATCC _____ (pRK5.tk.neo.hNRG3B1).

21. An expression vector according to claim 18 obtainable as ATCC _____ (pRK5.tk.neo.hNRG3B2).

22. A host cell comprising the vector of claim 18.

23. The host cell of claim 22 which is a mammalian cell.

24. The host cell of claim 23 which is a Chinese hamster ovary cell line.

25. A method for producing the amino acid sequence encoding an EGF-like domain that binds ErbB4 receptor, the method comprising:

- a) culturing a cell comprising the nucleic acid of claim 14; and
- b) recovering the polypeptide from the cell culture.

26. The method of claim 25 wherein the polypeptide is secreted into the culture medium and recovered from the culture medium.

27. An antibody that specifically binds to the polypeptide of claim 1.
28. A hybridoma cell line producing the antibody of claim 27.
29. An immunoadhesin comprising the polypeptide of claim 1 fused to an immunoglobulin sequence.
30. The immunoadhesin of claim 29, further comprising the EGF-like domain of SEQ ID NO:4.
31. The immunoadhesin of claim 29 wherein the immunoglobulin sequence is an immunoglobulin heavy chain constant domain sequence.
32. The immunoadhesin of claim 31 wherein the immunoglobulin sequence is a constant domain sequence of an IgG-1, IgG-2 or IgG-3.
33. A method of detecting an NRG3 in a sample, the method comprising:
- a) contacting the antibody of claim 27 with the sample;
 - b) detecting binding of the antibody to a polypeptide in the sample, wherein the polypeptide is an NRG3.
34. A method of detecting ErbB4 receptor in a sample, the method comprising:
- a) contacting the polypeptide of claim 1 with the sample; and
 - b) detecting binding of the amino acid sequence to a protein in the sample.
35. The method of claim 34 wherein the sample comprises a cell expressing ErbB4 receptor on its surface.
36. The method of claim 35 wherein the sample is a mammalian tissue sample.

37. A method of administering a NRG3 polypeptide to a mammal experiencing a disorder treatable with NRG3,
wherein the method comprises introducing into the mammal a cell comprising the nucleic acid of claim 14, and
wherein the NRG3 polypeptide is secreted by the cell.

38. The method of claim 37 wherein the cell is contained within a porous matrix and the matrix is administered to the mammal.

ABSTRACT OF THE INVENTION

The invention concerns a novel neuregulin related ligand (NRG3) including fragments and variants thereof, as new members of the neuregulin family of compounds. The invention also concerns methods and means for producing NRG3. The native polypeptides of the invention are characterized by containing an extracellular domain including an EGF-like domain, a transmembrane domain and a cytoplasmic domain. Isolated nucleotide sequences encoding such polypeptides, expression vectors containing the nucleotide sequences, recombinant host cells transformed with the vectors, and methods for the recombinant production for the novel NRG3s are also within the scope of the invention.

CCTGACCGGCCGGCGGCCCGGGCCGGTCTCGCCCCCTCTACCGAGCGCCTCGCCGCC
 CCCCCTCCCGGCCCGCGTCCCTCCCCCGTCTCTCCTCCCGCCCCGCCCGCCCTCTC
 GGGGGGAGGGGCGTGGGGGCAGGGAGCCGATTTGCATGCGGCCGCCGCGGCCGCTG
 CCTGAGCCGGAGCCCCGCCGCCGGAGCCCCGCGCCCCGCGCCCCGCGCCCCGCG
 CGGCCCCATGCCTCTGGCGCGGCCCTCGGGGGGGCGAAGGTGAAGATCGGCTCCTAG
 GATGAGTGAAGGGGGCGGCCGGTGCCTCGCCACCTGGTGCCGCTTCGGCAGCCGCCGC
 CTCAGCCGAGGAGGGCACCGCGGCGGCTGCGGCGGCGGCGGCGGCGGGCGGGGGCC
 CGGACGGCGGCGGAGAAGGGGGCGGCCGAACCCCCCGGGAGTTACGCTGTAGCGACT
 GCATCGTGTGGAACCGGCAGCAGACGTGGTTGTGCGTGGTGCCTCTGTTTCATCGGCTT
 CATCGGCCTGGGGCTCAGCCTCATGCTGCTTAAATGGATCGTGGTAGGCTCCGTCAAG
 GAGTACGTGCCACGGACCTGGTGGACTCCAAGGGAATGGGCCAGGACCCCTTCTTCC
 TCTCCAAGCCCAGCTCTTTCCCAAGGCTATGGAAACCACCAACAACCACTTCTACC
 ACGTCCCCCGCCACCCCTCTGCCGGCGGCGCCGCTTCTTCCAGGACGCCTAACCGGA
 TTAGCACCCGCTTGACCACCATCACACGGGCACCCACCCGCTTCCCTGGGCACCGGGT
 TCCCATCCGGGCTAGCCCGCGCTCTACCACAGCACGGAACACTGCTGCCCCCTCCGACG
 GTCCTGTCCACCACGGCCCCCTTTCTTCAGTAGCAGCACGCCCGGCTCCCGACCCCCGAT
 GCCAGGAGCCCCCAGTACGCAGGCGATGCCTTCTGGCCCACTGCGGCGTATGCTACC
 TCCTCCTACCTCCACGATTCCACTCCCTCCTGGACCCTGTCACCCCTTTCAGGATGCTGC
 TGCCGCTCTTCTCCTCCTCACCTCTTCCACCTCCTCCACTACCACCACCCAGAACTA
 GCACCAGCCCCAAATTTCACTACAACATACTCCACTGAACGATCTGAGCACTTCAA
 ACCCTGTGAGACAAGGACCTGGCGTATTGTCTCAATGATGGTGAATGCTTTGTGATT
 GAGACCCTGACAGGATCCCATAAAGCACTGTGCGTGCAAGGAAGGCTACCAAGGAGTC
 CGTTGTGATCAATTTCTGCCGAAAACAGACTCCATCTTATCGGATCCAACAGACCACTT
 GGGGATTGAATTCATGGAGAGTGAAGACGTTTATCAAAGGCAGGTGCTGTCAATTTCA
 TGTATCATCTTTGGAATTGTATCGTGGGCATGTTCTGTGCAGCATTCTACTTCAAAAG
 CAAGAAACAAGCTAAACAAATTCAGGAGCACCTGAAAGAGTCACAGAATGGGAAGAA
 CTACAGCCTCAAGGCATCCAGCACAAAGTCTGAGAGCTTGATGAAGAGCCATGTCCAT
 CTACAAAATTATTCAAAGGCGGATAGGCATCCTGTGACTGCGCTGGAGAAAATAATGG
 AGTCAAGTTTTTCAGCTCCCCAGTCGTTCCAGAAAGTCACTTCTCCTGACCGAGGAAG
 CCAGCCTATCAAGCACACAGCCAGGACAAAGGAGTGGGATGTTGCATAGGAATAC
 TTTCAGAAAGGGCACCAACCTCACCCCGAAGTCGACTGGGTGGTATTGTAGGACCAGCA
 TATCAACAACCTGAAGAATCAAGAATTCAGACCAGGATACGATACCTTGCCAAGGGA
 TAGAGGTGAGGAAGACTATATCCACCTGCCTATACAGCTGTGGTGTGTTGAAAGACC
 CCTGGACTTAAAGTATGTGTCCAATGGCTTAAGAACCCCAACAAAATGCATCAATAAAT
 ATGCAACTGCCTTCAAGAGAGACAAACCCCTATTTTAATAGCTTGGATCAAAGGACC
 TGGTGGGTTATTTATCCCAAGGGCCAATTCTGTGCCCATCATCCCGTCGATGGGTCTA
 GAAGAAACCTGCATGCAAATGCCAGGGATTTCTGACGTCAAAGCATTAAATGGTGCA
 AAAACTCCTACTCCGCTGACATTGTCAACGCGAGTATGCCAGTCAGTGATTGTCTTCTA
 GAAGAACAACAGGAAGTGAAAATATTACTAGAGACTGTGCAGGAACAGATCCGGATT
 CTGACTGATGCCAGACGGTCAGAAGACTTCGAACTGGCCAGCATGGAAACTGAGGAC
 AGTGCAGCGGAAAACACAGCCTTTCTCCCCCTGAGTCCCACGGCCAAATCAGAACGAG
 AGGCACAATTTGTCTTAAGAAATGAAATACAAAGAGACTCTGTGCTAACCAAGTGACT
 GGAAATGTAGGAATCTGTGCATTATATGCTTTGCTAAACAGGAAGGAGAGGAAATTA
 AATACAAATTATTTATATGCATTAATTTAAGAGCATCTACTTAGAAGCC

Figure 1

TCACCGACCTAGTGGACTCCACTAGGTCTGGTGGGCACGTA CTCTTGACGGAGCCCA
CACGATCCATTTGAGAAGCATGAGGCGCGGCCCATGCCTCTGCCGCGGCCCTCGGGG
GGGCGAAGGTGAANACCGGCTCCTAGGATGAGTGAAGGGGCGGCCGCTGCCTCGCCA
CCTGGTGGCGCTTCGGCAGCCGCCGCTCGGCCGAGGAGGGCACCGCGGCGGCTGCG
GCGGCGGCAGCGGCGGGCGGGGGCCCGACGGCGGCGGCGAAGGGGCGGCCGAGCC
CCCCCGGAGTTACGCTGTAGCGACTGCATCGTGTGGAACCGGCAGCAGACGTGGCT
GTGCGTGGTACCTCTGTTTCATCGGCTTCATCGGCCTGGGGCTCAGCCTCATGCTTCTCA
AATGGATCGTGGTGGGCTCCGTCAAGGAGTACGTGCCACCGACCTAGTGGACTCCAA
GGGGATGGGCCAGGACCCCTTCTTCTCTCCAAGCCCAGCTCTTTCCCCAAGGCCATG
GAGACCACCACCACTACCACTTCCACCACGTCCCCCGCCACCCCTCCGCCGGGGTG
CCGCCTCCTCCAGGACGCCCAACCGGATTAGCACTCGCCTGACCACCATCACGCGGGC
GCCACTCGCTTCCCCGGGCACCGGGTGCCCATCCGGGCCAGCCCGCGCTCCACCACA
GCACGGAACACTGCGGCCCTGCGACGGTCCCGTCCACCACGGCCCCGTTCTTCAGTA
GCAGCACGTGGGCTCCCGACCCCGGTGCCAGGAATCCAAGTACCCAGGCAATGCC
CTCCTGGCCTACTGCGGCATACGCTACCTCCTCTACCTTACGATTCTACTCCCTCCT
GGACCCTGTCTCCCTTTCAGGATGCTGCCTCCTCTTCTTCTCTCTCTCTCTCCGCTA
CCACCACCACACAGAACTAGCACCAGCCCCAAATTCATACGACGACATATCCAC
AGAGCGATCCGAGCACTTCAAACCCTGCCGAGACAAGGACCTTGCATACTGTCTCAAT
GATGGCGAGTGCTTTGTGATCGAAACCCTGACCGGATCCCATAAACTGTGCGGTGCA
AAGAAGGCTACCAAGGAGTCCGTTGTGATCAATTTCTGCCGAAAATGATTCCATCTT
ATCGGATCCAACAGACCACTTGGGGATTGAATTCATGGAGAGTGAAGAAGTTTATCAA
AGGCAGGTGCTGTCAATTTTCATGTATCATCTTTGGAATTGTCATCGTGGGCATGTTCTG
TGCAGCATTCTACTTCAAAGCAAGAAACAAGCTAAACAAATCCAAGAGCAGCTGAA
AGTGCCACAAAATGGTAAAAGCTACAGTCTCAAAGCATCCAGCACAATGGCAAAGTC
AGAGAACTTGGTGAAGAGCCATGTCCAGCTGCAAAATTATTCAAAGGTGGAAAGGCA
TCCTGTGACTGCATTGGAGAAAATGATGGAGTCAAGTTTTGTGCGCCCCCAGTCATTC
CCTGAGGTCCCTTCTCCTGACAGAGGAAGCCAGTCTGTCAAACACCACAGGAGTCTAT
CCTCTTGCTGCAGCCCAGGGCAAAGAAGTGGCATGCTCCATAGGAATGCCTTCAGAAG
GACACCCCGTCACCCCGAAGTAGGCTAGGTGGAATTGTGGGACCAGCATATCAGCA
ACTCGAAGAATCAAGGATCCCAGACCAGGATACGATACCTTGCCAAGGGATAGAGGT
CAGGAAGACTATATCCCACCTGCCTATACAGCTGTGGTGTGTTGAAAGACCCCTGGAC
TTAAAGTATTCATCCAGTGGTTTAAAAACCCAACGAAATACATCAATAAATATGCAAC
TGCCTTCAAGAGAGACAAACCCCTATTTTAATAGCTTGGAGCAAAGGACCTGGTGGG
CTATTCATCCACAAGGGCCAGTTCTGTGCCCATCATCCCTTCAGTGGGTTTAGAGGAA
ACCTGCCTGCAAATGCCAGGGATTTCTGAAGTCAAAGCATCAAATGGTGCAAAAACCT
CCTATTTCAGCTGACGTTGTCAATGTGAGTATTCCAGTCAGCGATTGTCTTATAGCAGA
ACAACAAGAAGTGAAAATATTGCTAGAACTGTCCAGGAGCAGATCCGAATTCTGACT
GATGCCAGACGGTCAGAAGACTACGAACTGGCCAGCGTAGAAACCGAGGACAGTGCA
AGCGAAAACACAGCCTTTCTCCCCCTGAGTCCCACAGCCAAATCAGAACGAGAGGGCGC
AATTTGTCTTAAGAAATGAAATACAAAGAGACTCTGCATTGACCAAGTGACTTGAGAT
GTAGGAATCTGTGCATTCTATGCTTTGCTCAACAGGAAAGAGAGGAAATCAAATACAA
ATTATTTATATGCATTAATTTAAGAGCATCTACTTAGAAGAAACCAATAGTCTATCGC
CCTCATATCATAGTGTTTTTTAACAAAATATTTTTTTAAGGGAAAGAAATGTTTCAGGA
GGGATAAAGCTT

Figure 2

ATGAGTGAAGGGGCGGCCGCTGCCTCGCCACCTGGTGCCGCTTCGGCAGCCG
 CCGCCTCGGCCGAGGAGGGCACCGCGGCGGCTGCGGCGGCGGCAGCGGCGG
 GCGGGGGCCCGGACGGCGGGCGGCGAAGGGGCGGCCGAGCCCCCGGGAGT
 TACGCTGTAGCGACTGCATCGTGTGGAACCGGCAGCAGACGTGGCTGTGCGT
 GGTACCTCTGTTTCATCGGCTTCATCGGCCTGGGGCTCAGCCTCATGCTTCTCA
 AATGGATCGTGGTGGGCTCCGTCAAGGAGTACGTGCCCCACCGACCTAGTGGA
 CTCCAAGGGGATGGGCCAGGACCCCTTCTTCTCTCCAAGCCCAGCTCTTTCC
 CCAAGGCCATGGAGACCACCACCACTACCACTTCCACCACGTCCCCCGCCACC
 CCCTCCGCGGGGGGTGCCGCTCCTCCAGGACGCCCAACCGGATTAGCACTCG
 CCTGACCACCATCACGCGGGCGCCCACTCGCTTCCCCGGGCACCGGGTGCCCA
 TCCGGGCCAGCCCGCGCTCCACCACAGCACGGAACACTGCGGCCCTGCGAC
 GGTCCCGTCCACCACGGCCCCGTTCTTCAGTAGCAGCACGCTGGGCTCCCGAC
 CCCCAGTGCCAGGAACCTCCAAGTACCCAGGCAATGCCCTCCTGGCCTACTGCG
 GCATACGCTACCTCCTCCTACCTTCACGATTCTACTCCCTCCTGGACCCTGTCT
 CCCTTTCAGGATGCTGCCTCCTCTTCTTCTCCTCCTCCTCCTCCGCTACCACC
 ACCACACCAGAACTAGCACCAGCCCCAAATTTTCATACGACGACATATTCCAC
 AGAGCGATCCGAGCACTTCAAACCCTGCCGAGACAAGGACCTTGCATACTGTC
 TCAATGATGGCGAGTGCTTTGTGATCGAAACCCTGACCGGATCCCATAAACAC
 TGTCGGTGCAAAGAAGGCTACCAAGGAGTCCGTTGTGATCAATTTCTGCCGAA
 AACTGATTCCATCTTATCGGATCCAACAGACCCTTGGGGATTGAATTCATGG
 AGAGTGAAGAAGTTTATCAAAGGCAGGTGCTGTCAATTTTCATGTATCATCTTT
 GGAATTGTTCATCGTGGGCATGTTCTGTGCAGCATTCTACTTCAAAGCAAGAA
 ACAAGCTAAACAAATCCAAGAGCAGCTGAAAGTGCCACAAAATGGTAAAAGC
 TACAGTCTCAAAGCATCCAGCACAAATGGCAAAGTCAGAGAACTTGGTGAAGA
 GCCATGTCCAGCTGCAAAATTATTCAAAGGTGGAAAGGCATCCTGTGACTGCA
 TTGGAGAAAATGATGGAGTCAAGTTTTGTGCGCCCCCAGTCATTCCCTGAGGT
 CCCTTCTCCTGACAGAGGAAGCCAGTCTGTCAAACACCACAGGAGTCTATCCT
 CTTGCTGCAGCCCAGGGCAAAGAAGTGGCATGCTCCATAGGAATGCCTTCAG
 AAGGACACCCCCGTCACCCCGAAGTAGGCTAGGTGGAATTGTGGGACCAGCA
 TATCAGCAACTCGAAGAATCAAGGATCCCAGACCAGGATACGATACCTTGCCA
 AGGGTATTTCATCCAGTGGTTTAAAAACCCAACGAAATACATCAATAAATATGC
 AACTGCCTTCAAGAGAGACAAACCCCTATTTTAATAGCTTGGAGCAAAAGGAC
 CTGGTGGGCTATTCATCCACAAGGGCCAGTTCTGTGCCCATCATCCCTTCAGT
 GGGTTTAGAGGAAACCTGCCTGCAAATGCCAGGGATTCTGAAGTCAAAGC
 ATCAAATGGTGCAAAAACCTCCTATTCAGCTGACGTTGTCAATGTGAGTATTCC
 AGTCAGCGATTGTCTTATAGCAGAACAAAGAAGTGAAAATATTGCTAGAA
 ACTGTCCAGGAGCAGATCCGAATTCTGACTGATGCCAGACGGTCAGAAGACT
 ACGAACTGGCCAGCGTAGAAACCGAGGACAGTGCAAGTGAAAACACAGCCTT
 TCTCCCCCTGAGTCCCACAGCCAAATCAGAACGAGAGGCGCAATTTGTCTTAA
 GAAATGAAATACAAAGAGACTCTGCATTGACCAAGTGA

Figure 3

hNRG3B1 1 MSEGAAASPPGAASAAAAAEEGTAAAAAAAAGGGPDGGGEGAAEPPR
mNRG3 1 MSEGAAASPPGAASAAAAAEEGTAAAAAAAAGGGPDGGGEGAAEPPR

hNRG3B1 51 ELRCSDCIVWNRQQTWLCVVPLFIGFIGLGLSLMLLKWIVVGSVKEYVPT
mNRG3 51 ELRCSDCIVWNRQQTWLCVVPLFIGFIGLGLSLMLLKWIVVGSVKEYVPT

hNRG3B1 101 DLVDSKGMGQDPFFLSKPSSFPAKAMTTTTTTSTTSPATPSAGGAASSRT
mNRG3 101 DLVDSKGMGQDPFFLSKPSSFPAKAMTTTTTTSTTSPATPSAGGAASSRT

hNRG3B1 151 PNRISTRLTTITRAPTRFPGHRVPIRASPRSTTARNTAAPATVPSTTAPF
mNRG3 151 PNRISTRLTTITRAPTRFPGHRVPIRASPRSTTARNTAAPATVLSSTTAPF

hNRG3B1 201 FSSSTLGSRPPVFGTPSTQAMPSWPTAAYATSSYLHDSTPSWTLSPFQD-
mNRG3 201 FSSSTPGSRPPMPGAPSTQAMPSWPTAAYATSSYLHDSTPSWTLSPFQDA

hNRG3B1 250 -AASSSSSSSSSAITTTTPETSTSPKFHTTYYSTERSEHFKEPDRDKLAYE
mNRG3 251 AAASSSSPSSSTSITTTTPETSTSPKFHTTYYSTERSEHFKEPDRDKLAYE

hNRG3B1 299 LNDGEQFV IETLTGSHKHQRKEGYQGVRQDQFLPKTDSILSDPTDHLGI
mNRG3 301 LNDGEQFV IETLTGSHKHQRKEGYQGVRQDQFLPKTDSILSDPTDHLGI

hNRG3B1 349 EFMESEEVYQROVLSISCIIFGIVIVGMFCAAFYFKSKKQAKQIQEQLKV
mNRG3 351 EFMESEEVYQROVLSISCIIFGIVIVGMFCAAFYFKSKKQAKQIQEHLKE

hNRG3B1 399 PONGKSYSLKASSTMAKSENLVKSHVQLQNYSKVERHPVTALEKMMESSF
mNRG3 401 SONGKNYSLKASST--KSESLVKSHVHLQNYSKADHPVTALEKIMESSF

hNRG3B1 449 VGQSFPEVPSPDRGSQSVKHHRSLSSCCSPGQRSGMLHRNAFRRTPPSP
mNRG3 449 SAQSFPEVTSPPDRGSQPIKHH-----SPGQRSGMLHRNTERRAPPSP

hNRG3B1 499 RSRLGGIVGPAYQQLSESRIPDDOTPCOGIEVRKTI SHLP IQLWCVERP
mNRG3 492 RSRLGGIVGPAYQQLSESRIPDDOTPCOGIEVRKTI SHLP IQLWCVERP

hNRG3B1 549 LDKYSSSGLKTRNTSINMQLPSRETNPFNSLEQKDLVGYSSSTRASSV
mNRG3 562 LDKYVSNGLRTOQNASINMQLPSRETNPFNSLDQKDLVGYLSPRANSSV

hNRG3B1 599 PIIPSVGLEETCLQMPGISVKS IKWCKNSYSADVNVSI PVSDCLIAEQ
mNRG3 592 PIIPSMGLEETCMQMPGISDKS IKWCKNSYSADIVNASMPVSDCVIEEQ

hNRG3B1 649 DEVKILLETVOEQIRILTDARRSEDFELASVETEDSASENTAFLPLSPTA
mNRG3 642 DEVKILLETVOEQIRILTDARRSEDFELASMETEDSASENTAFLPLSPTA

hNRG3B1 699 KSEREAQFVLRNEIQRDSALTK
mNRG3 692 KSEREAQFVLRNEIQRDSVLTKE

Figure 4A

hNRG3B1 1 MSEGAAAAASPPGAASAAAAASAEEGTAAAAAAAAGGGPDGGGEGAAEPPR
hNRG3B2 1 MSEGAAAAASPPGAASAAAAASAEEGTAAAAAAAAGGGPDGGGEGAAEPPR

hNRG3B1 51 ELRCSDCIVWNRQOTWLCVPLFIGFIGLGLSLMLLKWIVVGSVKEYVPT
hNRG3B2 51 ELRCSDCIVWNRQOTWLCVPLFIGFIGLGLSLMLLKWIVVGSVKEYVPT

hNRG3B1 101 DLVDSKGMGQDPFFLSKPSSFPKAMETTTTTTSTTSPATPSAGGAASSRT
hNRG3B2 101 DLVDSKGMGQDPFFLSKPSSFPKAMETTTTTTSTTSPATPSAGGAASSRT

hNRG3B1 151 PNRISTRLTTITRAPTRFPGHRVPIRASPRSTTARNTAAPATVPSTTAPF
hNRG3B2 151 PNRISTRLTTITRAPTRFPGHRVPIRASPRSTTARNTAAPATVPSTTAPF

hNRG3B1 201 FSSSTLGSRPPVPGTPTQAMPSWPTAAYATSSYLHDSTPSWTLSPPFODA
hNRG3B2 201 FSSSTLGSRPPVPGTPTQAMPSWPTAAYATSSYLHDSTPSWTLSPPFODA

hNRG3B1 251 ASSSSSSSSSATTITPETSTSPKFHTTTTSTERSEHFKPCRDKDLAYCLN
hNRG3B2 251 ASSSSSSSSSATTITPETSTSPKFHTTTTSTERSEHFKPCRDKDLAYCLN

hNRG3B1 301 DGEFCFVIETLTGSHKHCRCKEGYQGVRCDOFLPKTDSILSDPTDHLGIEF
hNRG3B2 301 DGEFCFVIETLTGSHKHCRCKEGYQGVRCDOFLPKTDSILSDPTDHLGIEF

hNRG3B1 351 MESEEVYORQVLSISCIIFGIVIVGMFCAAFYFKSKKOAKOIOEOLKVPO
hNRG3B2 351 MESEEVYORQVLSISCIIFGIVIVGMFCAAFYFKSKKOAKOIOEOLKVPO

hNRG3B1 401 NGKSYS LKASSTMAKSEN LVKSHVOLQNYSKVERHPVTALEKMMESSFVG
hNRG3B2 401 NGKSYS LKASSTMAKSEN LVKSHVOLQNYSKVERHPVTALEKMMESSFVG

hNRG3B1 451 POSFPEVPSPDRGSQSVKHHRLSSCCSPGQRSGMLHRNAFRRTPPSPRS
hNRG3B2 451 POSFPEVPSPDRGSQSVKHHRLSSCCSPGQRSGMLHRNAFRRTPPSPRS

hNRG3B1 501 RLGGIVGPAYQOLEESRIPDOOTIPCOG IEVRKTISHLP IQLWCVERPLD
hNRG3B2 501 RLGGIVGPAYQOLEESRIPDOOTIPCOG

hNRG3B1 551 LK YSSSGLKTORNTSINMQLPSRETNPFYFNSLEQKOLVGYSSTRASSVPI
hNRG3B2 529 - YSSSGLKTORNTSINMQLPSRETNPFYFNSLEQKOLVGYSSTRASSVPI

hNRG3B1 601 IPSVGLEETCLQMPGISEVKS IKWCKNSYSADVVNVSIPVSDCLIAEQQE
hNRG3B2 577 IPSVGLEETCLQMPGISEVKS IKWCKNSYSADVVNVSIPVSDCLIAEQQE

hNRG3B1 651 VKILLETVQEQIRILTDARRSEDYELASVETEDSASENTAFLPLSPTAKS
hNRG3B2 627 VKILLETVQEQIRILTDARRSEDYELASVETEDSASENTAFLPLSPTAKS

hNRG3B1 701 EREAQFVLRNEIQRDSALT K
hNRG3B2 677 EREAQFVLRNEIQRDSALT K

Figure 4B

hNRG3.egf	288	H	F	K	P	C	R	D	K	D	L	A	Y	C	L	N	D	G	E	C	F	V	I	E	T	L	T	G	S	H	K	H	-	C	R	C	K	E	G	Y	Q	G	V	R	C	-	D	Q	F	L
cARIA.egf	137	H	L	T	K	C	D	I	K	Q	K	A	F	C	V	N	G	G	E	C	Y	M	V	K	D	L	P	N	P	R	Y	L	C	R	C	P	N	E	F	T	G	D	R	C	-	Q	N	Y	V	
hAR.egf	142	K	K	N	P	C	N	A	E	F	Q	N	E	C	I	H	-	G	E	C	K	Y	I	E	H	L	E	A	V	T	-	-	-	C	K	C	Q	Q	E	Y	F	G	E	R	C	G	E	K	S	M
hBTC.egf	65	H	F	S	R	C	P	K	Q	Y	K	H	Y	C	I	K	-	G	R	C	R	F	V	V	A	E	Q	T	P	S	-	-	-	C	V	C	D	E	G	Y	I	G	A	R	C	E	R	V	D	L
hEGF.egf	972	S	D	S	E	C	P	L	S	H	D	G	Y	C	L	H	D	G	V	C	M	Y	I	E	A	L	D	K	Y	A	-	-	-	C	N	C	V	V	G	Y	I	G	E	R	C	Q	Y	R	D	L
hHB-EGF.egf	104	K	R	D	P	C	L	R	K	Y	K	D	F	C	I	H	-	G	E	C	K	Y	V	K	E	L	R	A	P	S	-	-	-	C	I	C	H	P	G	Y	H	G	E	R	C	H	G	L	S	L
hHRGα.egf	178	H	L	V	K	C	A	E	K	E	K	T	F	C	V	N	G	G	E	C	F	M	V	K	D	L	S	N	P	S	R	Y	L	C	K	C	Q	P	G	F	T	G	A	R	C	T	E	N	Y	P
hHRGβ.egf	178	H	L	V	K	C	A	E	K	E	K	T	F	C	V	N	G	G	E	C	F	M	V	K	D	L	S	N	P	S	R	Y	L	C	K	C	P	N	E	F	T	G	D	R	C	-	Q	N	Y	V
hTGFA.egf	43	H	F	N	D	C	P	D	S	H	T	Q	E	C	F	H	-	G	T	C	R	F	L	V	Q	E	D	K	P	A	-	-	-	C	V	C	H	S	G	Y	V	G	A	R	C	E	H	A	D	L
mEPR.egf	57	Q	I	T	K	C	S	S	D	M	D	G	Y	C	L	H	-	G	Q	C	I	Y	L	V	D	M	R	E	K	F	-	-	-	C	R	C	E	V	G	Y	T	G	L	R	C	E	H	F	F	L

Figure 5

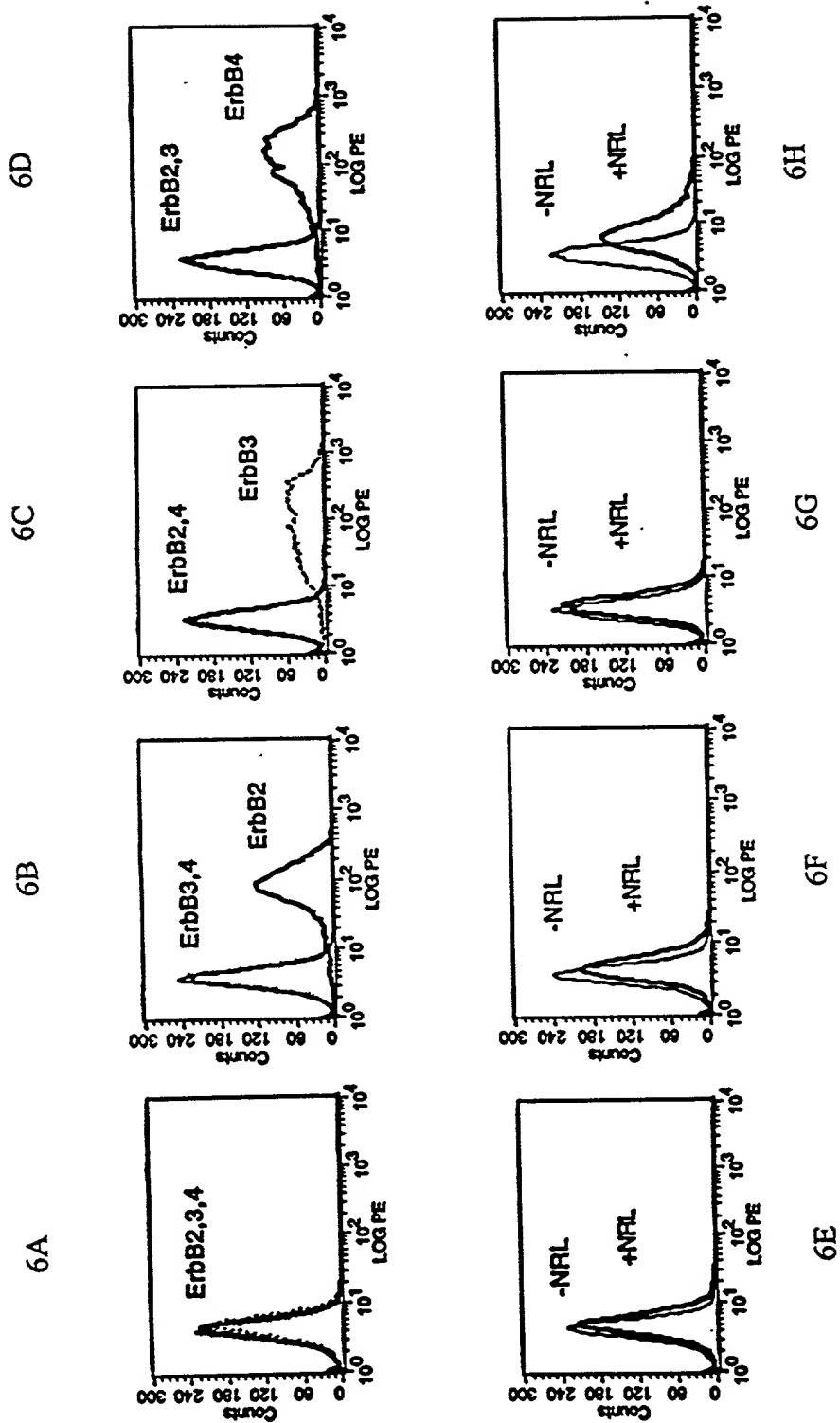


Figure 6A - 6H

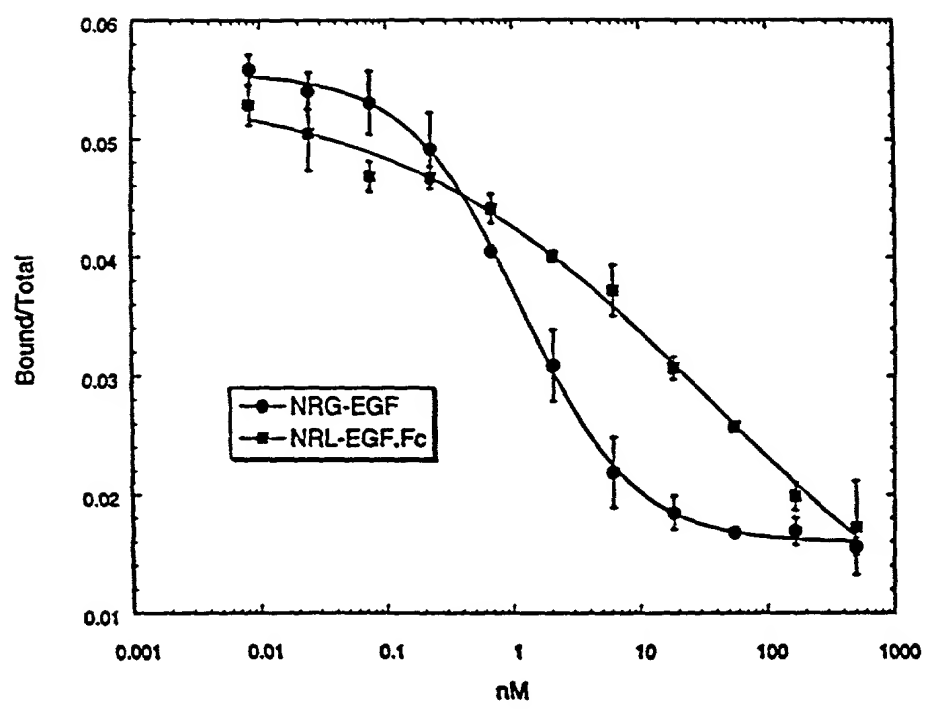


Figure 7

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ErbB4 RECEPTOR-SPECIFIC NEUREGULIN RELATED LIGANDS AND USES THEREFOR

the specification of which (check one) ☐ is attached hereto or ☒ was filed on 24 July 1997 as Application Serial No. 08/899,437 and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate have a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):

Priority Claimed
Yes No

Number	Country	Day/Month/Year Filed
--------	---------	----------------------

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below:

<u>60/052,019</u>	<u>July 9, 1997</u>
Application Ser. No.	Filing Date

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
----------------------	-------------	--------------------------------------

Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
----------------------	-------------	--------------------------------------

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

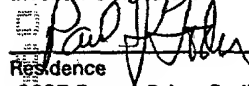
The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

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I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

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Date

Melanie Rose Mark

11/20/97

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Full name of third joint inventor, if any

Dong Xiao Zhang

Inventor's signature

Date

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Send correspondence to Genentech, Inc.

Attn: Deirdre L. Conley, Ph.D.
1 DNA Way
South San Francisco, CA 94080
Telephone: (650) 225-2066

I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

Full name of sole or first inventor

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Inventor's signature

Date

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Full name of second joint inventor, if any

Melanie Rose Mark

Inventor's signature

Date

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Full name of third joint inventor, if any

Dong Xiao Zhang

Inventor's signature

Date

Residence

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Citizenship

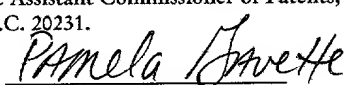
People's Republic of China

Post Office Address

1 DNA Way

South San Francisco, CA 94080

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<p>In re Application of</p> <p>Paul J. Godowski et al.</p> <p>Serial No.: Unassigned</p> <p>Filed: 11 January 2000</p> <p>For: ErbB4 RECEPTOR-SPECIFIC NEUREGULIN RELATED LIGANDS AND USES THEREFOR</p>	<p>Group Art Unit: Unassigned</p> <p>Examiner: Unassigned</p> <hr/> <p style="text-align: center;">CERTIFICATION UNDER 37 CFR 1.10</p> <p><u>EL 141 796 666 US</u> : Express Mail Number <u>11 January 2000</u> : Date of Deposit</p> <p>I hereby certify that this correspondence, consisting of attached papers, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.</p> <p style="text-align: center;"> Pamela Gavette</p>
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LETTER AND REQUEST TO USE COMPUTER-READABLE SEQUENCE LISTING
UNDER 37 CFR §1.821(e)

Assistant Commissioner of Patents
Washington, D.C. 20231

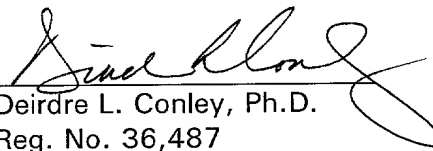
Sir:

Applicants respectfully request that the compliant computer-readable Sequence Listing filed in application Serial No. 08/899,437 be used as the computer-readable Sequence Listing for the present, above-identified application.

The paper copy of the Sequence Listing filed in the present application is identical to the computer-readable copy of the Sequence Listing filed in the application Serial No.

Respectfully submitted,
GENENTECH, INC.

Date: January 10, 2000

By: 
Deirdre L. Conley, Ph.D.
Reg. No. 36,487

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-2066
Fax: (650) 952-9881

08/899,437

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Godowski, Paul J., Mark, Melanie Rose, Zhang, Dong Xiao
- (ii) TITLE OF INVENTION: ErbB Receptor-Specific Neuregulin Related Ligands and Uses Therefor
- (iii) NUMBER OF SEQUENCES: 23
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 11-Jan-2000
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/899,437
(B) FILING DATE: 07/24/1997
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Conley, Deirdre L.
(B) REGISTRATION NUMBER: 36,487
(C) REFERENCE/DOCKET NUMBER: P1084R1C1
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650/225-2066
(B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2538 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
- (ix) FEATURE:
(A) NAME/KEY: mouse NRG3 nucleic acid
(B) LOCATION: 1-2538
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

TGGAATTGTC ATCGTGGGCA TGTTCGTGC AGCATTCTAC TTCAAAAGCA 1450
 AGAAACAAGC TAAACAAATT CAGGAGCACC TGAAAGAGTC ACAGAATGGG 1500
 AAGAACTACA GCCTCAAGGC ATCCAGCACA AAGTCTGAGA GCTTGATGAA 1550
 GAGCCATGTC CATCTACAAA ATTATTCAAA GGC GGATAGG CATCCTGTGA 1600
 CTGCGCTGGA GAAAATAATG GAGTCAAGTT TTTCAGCTCC CCAGTCGTTT 1650
 CCAGAAGTCA CTTCTCCTGA CCGAGGAAGC CAGCCTATCA AGCACCACAG 1700
 CCCAGGACAA AGGAGTGGGA TGTTCATAG GAATACTTTC AGAAGGGCAC 1750
 CACCCTCACC CCGAAGTCGA CTGGGTGGTA TTGTAGGACC AGCATATCAA 1800
 CAACTTGAAG AATCAAGAAT TCCAGACCAG GATACGATAC CTTGCCAAGG 1850
 GATAGAGGTC AGGAAGACTA TATCCACCT GCCTATACAG CTGTGGTGTG 1900
 TTGAAAGACC CCTGGACTTA AAGTATGTGT CCAATGGCTT AAGAACCCAA 1950
 CAAAATGCAT CAATAAATAT GCAACTGCCT TCAAGAGAGA CAAACCCCTA 2000
 TTTTAATAGC TTGGATCAAA AGGACCTGGT GGGTTATTTA TCCCCAAGGG 2050
 CCAATTCTGT GCCCATCATC CCGTCGATGG GTCTAGAAGA AACCTGCATG 2100
 CAAATGCCAG GGATTTCTGA CGTCAAAAGC ATTAAATGGT GCAAAAATC 2150
 CTACTCCGCT GACATTGTCA ACGCGAGTAT GCCAGTCAGT GATTGTCTTC 2200
 TAGAAGAACA ACAGGAAGTG AAAATATTAC TAGAGACTGT GCAGGAACAG 2250
 ATCCGGATTC TGAAGTATGC CAGACGGTCA GAAGACTTCG AACTGGCCAG 2300
 CATGGAAACT GAGGACAGTG CGAGCGAAAA CACAGCCTTT CTCCCCCTGA 2350
 GTCCACGGC CAAATCAGAA CGAGAGGCAC AATTTGTCTT AAGAAATGAA 2400
 ATACAAAGAG ACTCTGTGCT AACCAAGTGA CTGGAAATGT AGGAATCTGT 2450
 GCATTATATG CTTTGCTAAA CAGGAAGGAG AGGAAATTAA ATACAAATTA 2500
 TTTATATGCA TTAATTTAAG AGCATCTACT TAGAAGCC 2538

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 713 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: Mouse NRG3 (mNRG3)/amino acid seq.
- (B) LOCATION: 1-713
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Ser	Glu	Gly	Ala	Ala	Gly	Ala	Ser	Pro	Pro	Gly	Ala	Ala	Ser	
1				5					10					15	
Ala	Ala	Ala	Ala	Ser	Ala	Glu	Glu	Gly	Thr	Ala	Ala	Ala	Ala	Ala	
				20					25					30	
Ala	Ala	Ala	Ala	Gly	Gly	Gly	Pro	Asp	Gly	Gly	Gly	Glu	Gly	Ala	
				35					40					45	
Ala	Glu	Pro	Pro	Arg	Glu	Leu	Arg	Cys	Ser	Asp	Cys	Ile	Val	Trp	
				50					55					60	
Asn	Arg	Gln	Gln	Thr	Trp	Leu	Cys	Val	Val	Pro	Leu	Phe	Ile	Gly	
				65					70					75	
Phe	Ile	Gly	Leu	Gly	Leu	Ser	Leu	Met	Leu	Leu	Lys	Trp	Ile	Val	
				80					85					90	
Val	Gly	Ser	Val	Lys	Glu	Tyr	Val	Pro	Thr	Asp	Leu	Val	Asp	Ser	
				95					100					105	
Lys	Gly	Met	Gly	Gln	Asp	Pro	Phe	Phe	Leu	Ser	Lys	Pro	Ser	Ser	
				110					115					120	
Phe	Pro	Lys	Ala	Met	Glu	Thr	Thr	Thr	Thr	Thr	Thr	Ser	Thr	Thr	
				125					130					135	
Ser	Pro	Ala	Thr	Pro	Ser	Ala	Gly	Gly	Ala	Ala	Ser	Ser	Arg	Thr	
				140					145					150	
Pro	Asn	Arg	Ile	Ser	Thr	Arg	Leu	Thr	Thr	Ile	Thr	Arg	Ala	Pro	
				155					160					165	
Thr	Arg	Phe	Pro	Gly	His	Arg	Val	Pro	Ile	Arg	Ala	Ser	Pro	Arg	
				170					175					180	
Ser	Thr	Thr	Ala	Arg	Asn	Thr	Ala	Ala	Pro	Pro	Thr	Val	Leu	Ser	
				185					190					195	
Thr	Thr	Ala	Pro	Phe	Phe	Ser	Ser	Ser	Thr	Pro	Gly	Ser	Arg	Pro	
				200					205					210	
Pro	Met	Pro	Gly	Ala	Pro	Ser	Thr	Gln	Ala	Met	Pro	Ser	Trp	Pro	
				215					220					225	
Thr	Ala	Ala	Tyr	Ala	Thr	Ser	Ser	Tyr	Leu	His	Asp	Ser	Thr	Pro	
				230					235					240	
Ser	Trp	Thr	Leu	Ser	Pro	Phe	Gln	Asp	Ala	Ala	Ala	Ala	Ser	Ser	
				245					250					255	
Ser	Ser	Pro	Ser	Ser	Thr	Ser	Ser	Thr	Thr	Thr	Thr	Pro	Glu	Thr	
				260					265					270	
Ser	Thr	Ser	Pro	Lys	Phe	His	Thr	Thr	Thr	Tyr	Ser	Thr	Glu	Arg	
				275					280					285	

Ser	Glu	His	Phe	Lys	Pro	Cys	Arg	Asp	Lys	Asp	Leu	Ala	Tyr	Cys	290	295	300
Leu	Asn	Asp	Gly	Glu	Cys	Phe	Val	Ile	Glu	Thr	Leu	Thr	Gly	Ser	305	310	315
His	Lys	His	Cys	Arg	Cys	Lys	Glu	Gly	Tyr	Gln	Gly	Val	Arg	Cys	320	325	330
Asp	Gln	Phe	Leu	Pro	Lys	Thr	Asp	Ser	Ile	Leu	Ser	Asp	Pro	Thr	335	340	345
Asp	His	Leu	Gly	Ile	Glu	Phe	Met	Glu	Ser	Glu	Asp	Val	Tyr	Gln	350	355	360
Arg	Gln	Val	Leu	Ser	Ile	Ser	Cys	Ile	Ile	Phe	Gly	Ile	Val	Ile	365	370	375
Val	Gly	Met	Phe	Cys	Ala	Ala	Phe	Tyr	Phe	Lys	Ser	Lys	Lys	Gln	380	385	390
Ala	Lys	Gln	Ile	Gln	Glu	His	Leu	Lys	Glu	Ser	Gln	Asn	Gly	Lys	395	400	405
Asn	Tyr	Ser	Leu	Lys	Ala	Ser	Ser	Thr	Lys	Ser	Glu	Ser	Leu	Met	410	415	420
Lys	Ser	His	Val	His	Leu	Gln	Asn	Tyr	Ser	Lys	Ala	Asp	Arg	His	425	430	435
Pro	Val	Thr	Ala	Leu	Glu	Lys	Ile	Met	Glu	Ser	Ser	Phe	Ser	Ala	440	445	450
Pro	Gln	Ser	Phe	Pro	Glu	Val	Thr	Ser	Pro	Asp	Arg	Gly	Ser	Gln	455	460	465
Pro	Ile	Lys	His	His	Ser	Pro	Gly	Gln	Arg	Ser	Gly	Met	Leu	His	470	475	480
Arg	Asn	Thr	Phe	Arg	Arg	Ala	Pro	Pro	Ser	Pro	Arg	Ser	Arg	Leu	485	490	495
Gly	Gly	Ile	Val	Gly	Pro	Ala	Tyr	Gln	Gln	Leu	Glu	Glu	Ser	Arg	500	505	510
Ile	Pro	Asp	Gln	Asp	Thr	Ile	Pro	Cys	Gln	Gly	Ile	Glu	Val	Arg	515	520	525
Lys	Thr	Ile	Ser	His	Leu	Pro	Ile	Gln	Leu	Trp	Cys	Val	Glu	Arg	530	535	540
Pro	Leu	Asp	Leu	Lys	Tyr	Val	Ser	Asn	Gly	Leu	Arg	Thr	Gln	Gln	545	550	555
Asn	Ala	Ser	Ile	Asn	Met	Gln	Leu	Pro	Ser	Arg	Glu	Thr	Asn	Pro	560	565	570

Tyr Phe Asn Ser Leu Asp Gln Lys Asp Leu Val Gly Tyr Leu Ser
 575 580 585
 Pro Arg Ala Asn Ser Val Pro Ile Ile Pro Ser Met Gly Leu Glu
 590 595 600
 Glu Thr Cys Met Gln Met Pro Gly Ile Ser Asp Val Lys Ser Ile
 605 610 615
 Lys Trp Cys Lys Asn Ser Tyr Ser Ala Asp Ile Val Asn Ala Ser
 620 625 630
 Met Pro Val Ser Asp Cys Leu Leu Glu Glu Gln Gln Glu Val Lys
 635 640 645
 Ile Leu Leu Glu Thr Val Gln Glu Gln Ile Arg Ile Leu Thr Asp
 650 655 660
 Ala Arg Arg Ser Glu Asp Phe Glu Leu Ala Ser Met Glu Thr Glu
 665 670 675
 Asp Ser Ala Ser Glu Asn Thr Ala Phe Leu Pro Leu Ser Pro Thr
 680 685 690
 Ala Lys Ser Glu Arg Glu Ala Gln Phe Val Leu Arg Asn Glu Ile
 695 700 705
 Gln Arg Asp Ser Val Leu Thr Lys
 710 713

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 362 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: mNRG3 extracellular domainAmino acid seq
- (B) LOCATION: 1-362
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ser Glu Gly Ala Ala Gly Ala Ser Pro Pro Gly Ala Ala Ser
 1 5 10 15
 Ala Ala Ala Ala Ser Ala Glu Glu Gly Thr Ala Ala Ala Ala Ala
 20 25 30
 Ala Ala Ala Ala Gly Gly Gly Pro Asp Gly Gly Gly Glu Gly Ala
 35 40 45
 Ala Glu Pro Pro Arg Glu Leu Arg Cys Ser Asp Cys Ile Val Trp
 50 55 60

Asn	Arg	Gln	Gln	Thr 65	Trp	Leu	Cys	Val	Val 70	Pro	Leu	Phe	Ile	Gly 75
Phe	Ile	Gly	Leu	Gly 80	Leu	Ser	Leu	Met	Leu 85	Leu	Lys	Trp	Ile	Val 90
Val	Gly	Ser	Val	Lys 95	Glu	Tyr	Val	Pro	Thr 100	Asp	Leu	Val	Asp	Ser 105
Lys	Gly	Met	Gly	Gln 110	Asp	Pro	Phe	Phe	Leu 115	Ser	Lys	Pro	Ser	Ser 120
Phe	Pro	Lys	Ala	Met 125	Glu	Thr	Thr	Thr	Thr 130	Thr	Thr	Ser	Thr	Thr 135
Ser	Pro	Ala	Thr	Pro 140	Ser	Ala	Gly	Gly	Ala 145	Ala	Ser	Ser	Arg	Thr 150
Pro	Asn	Arg	Ile	Ser 155	Thr	Arg	Leu	Thr	Thr 160	Ile	Thr	Arg	Ala	Pro 165
Thr	Arg	Phe	Pro	Gly 170	His	Arg	Val	Pro	Ile 175	Arg	Ala	Ser	Pro	Arg 180
Ser	Thr	Thr	Ala	Arg 185	Asn	Thr	Ala	Ala	Pro 190	Pro	Thr	Val	Leu	Ser 195
Thr	Thr	Ala	Pro	Phe 200	Phe	Ser	Ser	Ser	Thr 205	Pro	Gly	Ser	Arg	Pro 210
Pro	Met	Pro	Gly	Ala 215	Pro	Ser	Thr	Gln	Ala 220	Met	Pro	Ser	Trp	Pro 225
Thr	Ala	Ala	Tyr	Ala 230	Thr	Ser	Ser	Tyr	Leu 235	His	Asp	Ser	Thr	Pro 240
Ser	Trp	Thr	Leu	Ser 245	Pro	Phe	Gln	Asp	Ala 250	Ala	Ala	Ala	Ser	Ser 255
Ser	Ser	Pro	Ser	Ser 260	Thr	Ser	Ser	Thr	Thr 265	Thr	Thr	Pro	Glu	Thr 270
Ser	Thr	Ser	Pro	Lys 275	Phe	His	Thr	Thr	Thr 280	Tyr	Ser	Thr	Glu	Arg 285
Ser	Glu	His	Phe	Lys 290	Pro	Cys	Arg	Asp	Lys 295	Asp	Leu	Ala	Tyr	Cys 300
Leu	Asn	Asp	Gly	Glu 305	Cys	Phe	Val	Ile	Glu 310	Thr	Leu	Thr	Gly	Ser 315
His	Lys	His	Cys	Arg 320	Cys	Lys	Glu	Gly	Tyr 325	Gln	Gly	Val	Arg	Cys 330
Asp	Gln	Phe	Leu	Pro 335	Lys	Thr	Asp	Ser	Ile 340	Leu	Ser	Asp	Pro	Thr 345

Asp His Leu Gly Ile Glu Phe Met Glu Ser Glu Asp Val Tyr Gln
 350 355 360

Arg Gln
 362

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 47 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: NRG3 EGF-like domain/amino acid seq.
- (B) LOCATION: 1-47
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

His Phe Lys Pro Cys Arg Asp Lys Asp Leu Ala Tyr Cys Leu Asn
 1 5 10 15
 Asp Gly Glu Cys Phe Val Ile Glu Thr Leu Thr Gly Ser His Lys
 20 25 30
 His Cys Arg Cys Lys Glu Gly Tyr Gln Gly Val Arg Cys Asp Gln
 35 40 45
 Phe Leu
 47

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2502 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: Human NRG3B1(hNRG3B1)/nucleic acid seq.
- (B) LOCATION: 1-2502
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCACCGACCT AGTGGACTCC ACTAGGTCGG TGGGCACGTA CTCCTTGACG 50
 GAGCCCACCA CGATCCATTT GAGAAGCATG AGGCCGCGGCC CCATGCCTCT 100
 GCCGCGGCCC TCGGGGGGGC GAAGGTGAAN ACCGGCTCCT AGGATGAGTG 150
 AAGGGGCGGC CGCTGCCTCG CCACCTGGTG CCGCTTCGGC AGCCGCCGCC 200

TCGGCCGAGG AGGGCACCGC GGCGGCTGCG GCGGCGGCAG CGGCGGGCGG 250
GGGCCCCGAC GGCGGCGGCG AAGGGGCGGC CGAGCCCCCC CGGGAGTTAC 300
GCTGTAGCGA CTGCATCGTG TGGAACCGGC AGCAGACGTG GCTGTGCGTG 350
GTACCTCTGT TCATCGGCTT CATCGGCCTG GGGCTCAGCC TCATGCTTCT 400
CAAATGGATC GTGGTGGGCT CCGTCAAGGA GTACGTGCCC ACCGACCTAG 450
TGGACTCCAA GGGGATGGGC CAGGACCCCT TCTTCCTCTC CAAGCCCAGC 500
TCTTTCCCCA AGGCCATGGA GACCACCACC ACTACCACTT CCACCACGTC 550
CCCCGCCACC CCTCCGCCG GGGGTGCCGC CTCCTCCAGG ACGCCCAACC 600
GGATTAGCAC TCGCCTGACC ACCATCACGC GGGCGCCCAC TCGCTTCCCC 650
GGGCACCGGG TGCCCATCCG GGCCAGCCCG CGCTCCACCA CAGCACGGAA 700
CACTGCGGCC CCTGCGACGG TCCCGTCCAC CACGGCCCCG TTCTTCAGTA 750
GCAGCACGCT GGGCTCCCGA CCCCCGGTGC CAGGAACTCC AAGTACCCAG 800
GCAATGCCCT CCTGGCCTAC TGCGGCATAC GCTACCTCCT CCTACCTTCA 850
CGATTCTACT CCTCCTGGA CCCTGTCTCC CTTTCAGGAT GCTGCCTCCT 900
CTTCTTCCTC TTCTTCCTCC TCCGCTACCA CCACCACACC AGAAACTAGC 950
ACCAGCCCCA AATTTCATAC GACGACATAT TCCACAGAGC GATCCGAGCA 1000
CTTCAAACCC TGCCGAGACA AGGACCTTGC ATACTGTCTC AATGATGGCG 1050
AGTGCTTTGT GATCGAAACC CTGACCGGAT CCCATAAACA CTGTGCGGTGC 1100
AAAGAAGGCT ACCAAGGAGT CCGTTGTGAT CAATTTCTGC CGAAAAGTGA 1150
TTCCATCTTA TCGGATCCAA CAGACCACTT GGGGATTGAA TTCATGGAGA 1200
GTGAAGAAGT TTATCAAAGG CAGGTGCTGT CAATTTCATG TATCATCTTT 1250
GGAATTGTCA TCGTGGGCAT GTTCTGTGCA GCATTCTACT TCAAAAGCAA 1300
GAAACAAGCT AAACAAATCC AAGAGCAGCT GAAAGTGCCA CAAAATGGTA 1350
AAAGCTACAG TCTCAAAGCA TCCAGCACAA TGGCAAAGTC AGAGAACTTG 1400
GTGAAGAGCC ATGTCCAGCT GCAAAATTAT TCAAAGGTGG AAAGGCATCC 1450
TGTGACTGCA TTGGAGAAAA TGATGGAGTC AAGTTTTGTC GGCCCCCAGT 1500
CATTCCCTGA GGTCCCTTCT CCTGACAGAG GAAGCCAGTC GTCAAACAC 1550
CACAGGAGTC TATCCTCTTG CTGCAGCCCA GGGCAAAGAA GTGGCATGCT 1600
CCATAGGAAT GCCTTCAGAA GGACACCCCC GTCACCCCGA AGTAGGCTAG 1650

GTGGAATTGT GGGACCAGCA TATCAGCAAC TCGAAGAATC AAGGATCCCA 1700
 GACCAGGATA CGATACCTTG CCAAGGGATA GAGGTCAGGA AGACTATATC 1750
 CCACCTGCCT ATACAGCTGT GGTGTGTTGA AAGACCCCTG GACTTAAAGT 1800
 ATTCATCCAG TGGTTTAAAA ACCCAACGAA ATACATCAAT AAATATGCAA 1850
 CTGCCTTCAA GAGAGACAAA CCCCTATTTT AATAGCTTGG AGCAAAAGGA 1900
 CCTGGTGGGC TATTCATCCA CAAGGGCCAG TTCTGTGCCC ATCATCCCTT 1950
 CAGTGGGTTT AGAGGAAACC TGCCTGCAAA TGCCAGGGAT TTCTGAAGTC 2000
 AAAAGCATCA AATGGTGCAA AAACCTCTAT TCAGCTGACG TTGTCAATGT 2050
 GAGTATTCCA GTCAGCGATT GTCTTATAGC AGAACAACAA GAAGTGAAAA 2100
 TATTGCTAGA AACTGTCCAG GAGCAGATCC GAATTCTGAC TGATGCCAGA 2150
 CGGTCAGAAG ACTACGAACT GGCCAGCGTA GAAACCGAGG ACAGTGCAAG 2200
 CGAAAACACA GCCTTTCTCC CCCTGAGTCC CACAGCCAAA TCAGAACGAG 2250
 AGGCGCAATT TGTCTTAAGA AATGAAATAC AAAGAGACTC TGCATTGACC 2300
 AAGTGACTTG AGATGTAGGA ATCTGTGCAT TCTATGCTTT GCTCAACAGG 2350
 AAAGAGAGGA AATCAAATAC AAATTATTTA TATGCATTAA TTTAAGAGCA 2400
 TCTACTTAGA AGAAACCAAA TAGTCTATCG CCCTCATATC ATAGTGTTTT 2450
 TTAACAAAAT ATTTTTTTAA GGGAAAGAAA TGTTTCAGGA GGGATAAAGC 2500
 TT 2502

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 720 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: hNRG3B1 amino acid sequence
- (B) LOCATION: 1-720
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Ser	Glu	Gly	Ala	Ala	Ala	Ala	Ser	Pro	Pro	Gly	Ala	Ala	Ser
1				5				10						15
Ala	Ala	Ala	Ala	Ser	Ala	Glu	Glu	Gly	Thr	Ala	Ala	Ala	Ala	Ala
				20				25						30

Ala	Ala	Ala	Ala	Gly	Gly	Gly	Pro	Asp	Gly	Gly	Gly	Glu	Gly	Ala		35	40	45
Ala	Glu	Pro	Pro	Arg	Glu	Leu	Arg	Cys	Ser	Asp	Cys	Ile	Val	Trp		50	55	60
Asn	Arg	Gln	Gln	Thr	Trp	Leu	Cys	Val	Val	Pro	Leu	Phe	Ile	Gly		65	70	75
Phe	Ile	Gly	Leu	Gly	Leu	Ser	Leu	Met	Leu	Leu	Lys	Trp	Ile	Val		80	85	90
Val	Gly	Ser	Val	Lys	Glu	Tyr	Val	Pro	Thr	Asp	Leu	Val	Asp	Ser		95	100	105
Lys	Gly	Met	Gly	Gln	Asp	Pro	Phe	Phe	Leu	Ser	Lys	Pro	Ser	Ser		110	115	120
Phe	Pro	Lys	Ala	Met	Glu	Thr	Thr	Thr	Thr	Thr	Thr	Ser	Thr	Thr		125	130	135
Ser	Pro	Ala	Thr	Pro	Ser	Ala	Gly	Gly	Ala	Ala	Ser	Ser	Arg	Thr		140	145	150
Pro	Asn	Arg	Ile	Ser	Thr	Arg	Leu	Thr	Thr	Ile	Thr	Arg	Ala	Pro		155	160	165
Thr	Arg	Phe	Pro	Gly	His	Arg	Val	Pro	Ile	Arg	Ala	Ser	Pro	Arg		170	175	180
Ser	Thr	Thr	Ala	Arg	Asn	Thr	Ala	Ala	Pro	Ala	Thr	Val	Pro	Ser		185	190	195
Thr	Thr	Ala	Pro	Phe	Phe	Ser	Ser	Ser	Thr	Leu	Gly	Ser	Arg	Pro		200	205	210
Pro	Val	Pro	Gly	Thr	Pro	Ser	Thr	Gln	Ala	Met	Pro	Ser	Trp	Pro		215	220	225
Thr	Ala	Ala	Tyr	Ala	Thr	Ser	Ser	Tyr	Leu	His	Asp	Ser	Thr	Pro		230	235	240
Ser	Trp	Thr	Leu	Ser	Pro	Phe	Gln	Asp	Ala	Ala	Ser	Ser	Ser	Ser		245	250	255
Ser	Ser	Ser	Ser	Ser	Ala	Thr	Thr	Thr	Thr	Pro	Glu	Thr	Ser	Thr		260	265	270
Ser	Pro	Lys	Phe	His	Thr	Thr	Thr	Tyr	Ser	Thr	Glu	Arg	Ser	Glu		275	280	285
His	Phe	Lys	Pro	Cys	Arg	Asp	Lys	Asp	Leu	Ala	Tyr	Cys	Leu	Asn		290	295	300
Asp	Gly	Glu	Cys	Phe	Val	Ile	Glu	Thr	Leu	Thr	Gly	Ser	His	Lys		305	310	315

His	Cys	Arg	Cys	Lys 320	Glu	Gly	Tyr	Gln	Gly 325	Val	Arg	Cys	Asp	Gln 330
Phe	Leu	Pro	Lys	Thr 335	Asp	Ser	Ile	Leu	Ser 340	Asp	Pro	Thr	Asp	His 345
Leu	Gly	Ile	Glu	Phe 350	Met	Glu	Ser	Glu	Glu 355	Val	Tyr	Gln	Arg	Gln 360
Val	Leu	Ser	Ile	Ser 365	Cys	Ile	Ile	Phe	Gly 370	Ile	Val	Ile	Val	Gly 375
Met	Phe	Cys	Ala	Ala 380	Phe	Tyr	Phe	Lys	Ser 385	Lys	Lys	Gln	Ala	Lys 390
Gln	Ile	Gln	Glu	Gln 395	Leu	Lys	Val	Pro	Gln 400	Asn	Gly	Lys	Ser	Tyr 405
Ser	Leu	Lys	Ala	Ser 410	Ser	Thr	Met	Ala	Lys 415	Ser	Glu	Asn	Leu	Val 420
Lys	Ser	His	Val	Gln 425	Leu	Gln	Asn	Tyr	Ser 430	Lys	Val	Glu	Arg	His 435
Pro	Val	Thr	Ala	Leu 440	Glu	Lys	Met	Met	Glu 445	Ser	Ser	Phe	Val	Gly 450
Pro	Gln	Ser	Phe	Pro 455	Glu	Val	Pro	Ser	Pro 460	Asp	Arg	Gly	Ser	Gln 465
Ser	Val	Lys	His	His 470	Arg	Ser	Leu	Ser	Ser 475	Cys	Cys	Ser	Pro	Gly 480
Gln	Arg	Ser	Gly	Met 485	Leu	His	Arg	Asn	Ala 490	Phe	Arg	Arg	Thr	Pro 495
Pro	Ser	Pro	Arg	Ser 500	Arg	Leu	Gly	Gly	Ile 505	Val	Gly	Pro	Ala	Tyr 510
Gln	Gln	Leu	Glu	Glu 515	Ser	Arg	Ile	Pro	Asp 520	Gln	Asp	Thr	Ile	Pro 525
Cys	Gln	Gly	Ile	Glu 530	Val	Arg	Lys	Thr	Ile 535	Ser	His	Leu	Pro	Ile 540
Gln	Leu	Trp	Cys	Val 545	Glu	Arg	Pro	Leu	Asp 550	Leu	Lys	Tyr	Ser	Ser 555
Ser	Gly	Leu	Lys	Thr 560	Gln	Arg	Asn	Thr	Ser 565	Ile	Asn	Met	Gln	Leu 570
Pro	Ser	Arg	Glu	Thr 575	Asn	Pro	Tyr	Phe	Asn 580	Ser	Leu	Glu	Gln	Lys 585
Asp	Leu	Val	Gly	Tyr 590	Ser	Ser	Thr	Arg	Ala 595	Ser	Ser	Val	Pro	Ile 600

Ile	Pro	Ser	Val	Gly	Leu	Glu	Glu	Thr	Cys	Leu	Gln	Met	Pro	Gly
				605					610					615
Ile	Ser	Glu	Val	Lys	Ser	Ile	Lys	Trp	Cys	Lys	Asn	Ser	Tyr	Ser
				620					625					630
Ala	Asp	Val	Val	Asn	Val	Ser	Ile	Pro	Val	Ser	Asp	Cys	Leu	Ile
				635					640					645
Ala	Glu	Gln	Gln	Glu	Val	Lys	Ile	Leu	Leu	Glu	Thr	Val	Gln	Glu
				650					655					660
Gln	Ile	Arg	Ile	Leu	Thr	Asp	Ala	Arg	Arg	Ser	Glu	Asp	Tyr	Glu
				665					670					675
Leu	Ala	Ser	Val	Glu	Thr	Glu	Asp	Ser	Ala	Ser	Glu	Asn	Thr	Ala
				680					685					690
Phe	Leu	Pro	Leu	Ser	Pro	Thr	Ala	Lys	Ser	Glu	Arg	Glu	Ala	Gln
				695					700					705
Phe	Val	Leu	Arg	Asn	Glu	Ile	Gln	Arg	Asp	Ser	Ala	Leu	Thr	Lys
				710					715					720

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 360 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: hNRG3 extracellular domain/Amino AcidSeq
- (B) LOCATION: 1-360
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met	Ser	Glu	Gly	Ala	Ala	Ala	Ala	Ser	Pro	Pro	Gly	Ala	Ala	Ser
1				5					10					15
Ala	Ala	Ala	Ala	Ser	Ala	Glu	Glu	Gly	Thr	Ala	Ala	Ala	Ala	Ala
				20					25					30
Ala	Ala	Ala	Ala	Gly	Gly	Gly	Pro	Asp	Gly	Gly	Gly	Glu	Gly	Ala
				35					40					45
Ala	Glu	Pro	Pro	Arg	Glu	Leu	Arg	Cys	Ser	Asp	Cys	Ile	Val	Trp
				50					55					60
Asn	Arg	Gln	Gln	Thr	Trp	Leu	Cys	Val	Val	Pro	Leu	Phe	Ile	Gly
				65					70					75
Phe	Ile	Gly	Leu	Gly	Leu	Ser	Leu	Met	Leu	Leu	Lys	Trp	Ile	Val
				80					85					90

Val Gly Ser Val	Lys Glu Tyr Val	Pro Thr Asp Leu Val	Asp Ser
	95	100	105
Lys Gly Met Gly	Gln Asp Pro Phe Phe	Leu Ser Lys Pro Ser	Ser
	110	115	120
Phe Pro Lys Ala	Met Glu Thr Thr Thr	Thr Thr Thr Ser Thr	Thr
	125	130	135
Ser Pro Ala Thr	Pro Ser Ala Gly Gly	Ala Ala Ser Ser Arg	Thr
	140	145	150
Pro Asn Arg Ile	Ser Thr Arg Leu Thr	Thr Ile Thr Arg Ala	Pro
	155	160	165
Thr Arg Phe Pro	Gly His Arg Val Pro	Ile Arg Ala Ser Pro	Arg
	170	175	180
Ser Thr Thr Ala	Arg Asn Thr Ala Ala	Pro Ala Thr Val Pro	Ser
	185	190	195
Thr Thr Ala Pro	Phe Phe Ser Ser Ser	Thr Leu Gly Ser Arg	Pro
	200	205	210
Pro Val Pro Gly	Thr Pro Ser Thr Gln	Ala Met Pro Ser Trp	Pro
	215	220	225
Thr Ala Ala Tyr	Ala Thr Ser Ser Tyr	Leu His Asp Ser Thr	Pro
	230	235	240
Ser Trp Thr Leu	Ser Pro Phe Gln Asp	Ala Ala Ser Ser Ser	Ser
	245	250	255
Ser Ser Ser Ser	Ser Ala Thr Thr Thr	Thr Pro Glu Thr Ser	Thr
	260	265	270
Ser Pro Lys Phe	His Thr Thr Thr Tyr	Ser Thr Glu Arg Ser	Glu
	275	280	285
His Phe Lys Pro	Cys Arg Asp Lys Asp	Leu Ala Tyr Cys Leu	Asn
	290	295	300
Asp Gly Glu Cys	Phe Val Ile Glu Thr	Leu Thr Gly Ser His	Lys
	305	310	315
His Cys Arg Cys	Lys Glu Gly Tyr Gln	Gly Val Arg Cys Asp	Gln
	320	325	330
Phe Leu Pro Lys	Thr Asp Ser Ile Leu	Ser Asp Pro Thr Asp	His
	335	340	345
Leu Gly Ile Glu	Phe Met Glu Ser Glu	Glu Val Tyr Gln Arg	Gln
	350	355	360

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

- (ix) FEATURE:
(A) NAME/KEY: NRG3 EGF-like domain/amino acid seq.
(B) LOCATION: 1-47
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

His Phe Lys Pro Cys Arg Asp Lys Asp Leu Ala Tyr Cys Leu Asn
1 5 10 15
Asp Gly Glu Cys Phe Val Ile Glu Thr Leu Thr Gly Ser His Lys
20 25 30
His Cys Arg Cys Lys Glu Gly Tyr Gln Gly Val Arg Cys Asp Gln
35 40 45
Phe Leu
47

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

- (ix) FEATURE:
(A) NAME/KEY: cARIA.egf
(B) LOCATION: 1-48
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

His Leu Thr Lys Cys Asp Ile Lys Gln Lys Ala Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Tyr Met Val Lys Asp Leu Pro Asn Pro Pro Arg
20 25 30
Tyr Leu Cys Arg Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln
35 40 45
Asn Tyr Val
48

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: hAR.egf
(B) LOCATION: 1-45
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys Lys Asn Pro Cys Asn Ala Glu Phe Gln Asn Phe Cys Ile His
1 5 10 15
Gly Glu Cys Lys Tyr Ile Glu His Leu Glu Ala Val Thr Cys Lys
20 25 30
Cys Gln Gln Glu Tyr Phe Gly Glu Arg Cys Gly Glu Lys Ser Met
35 40 45

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: hBTC.efg
(B) LOCATION: 1-45
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

His Phe Ser Arg Cys Pro Lys Gln Tyr Lys His Tyr Cys Ile Lys
1 5 10 15
Gly Arg Cys Arg Phe Val Val Ala Glu Gln Thr Pro Ser Cys Val
20 25 30
Cys Asp Glu Gly Tyr Ile Gly Ala Arg Cys Glu Arg Val Asp Leu
35 40 45

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: hEGF.egf
(B) LOCATION: 1-46

- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Ser Asp Ser Glu Cys Pro Leu Ser His Asp Gly Tyr Cys Leu His
 1           5           10           15
Asp Gly Val Cys Met Tyr Ile Glu Ala Leu Asp Lys Tyr Ala Cys
          20           25           30
Asn Cys Val Val Gly Tyr Ile Gly Glu Arg Cys Gln Tyr Arg Asp
          35           40           45
Leu
46

```

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: hHB-EGF.egf
- (B) LOCATION: 1-45
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

Lys Arg Asp Pro Cys Leu Arg Lys Tyr Lys Asp Phe Cys Ile His
 1           5           10           15
Gly Glu Cys Lys Tyr Val Lys Glu Leu Arg Ala Pro Ser Cys Ile
          20           25           30
Cys His Pro Gly Tyr His Gly Glu Arg Cys His Gly Leu Ser Leu
          35           40           45

```

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 49 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: hHRGalpha.egf
- (B) LOCATION: 1-49
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```

His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
 1           5           10           15

```


Cys His Ser Gly Tyr Val Gly Ala Arg Cys Glu His Ala Asp Leu
 35 40 45

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

- (ix) FEATURE:
 (A) NAME/KEY: mEPR.egf
 (B) LOCATION: 1-45
 (C) IDENTIFICATION METHOD:
 (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gln Ile Thr Lys Cys Ser Ser Asp Met Asp Gly Tyr Cys Leu His
 1 5 10 15
 Gly Gln Cys Ile Tyr Leu Val Asp Met Arg Glu Lys Phe Cys Arg
 20 25 30
 Cys Glu Val Gly Tyr Thr Gly Leu Arg Cys Glu His Phe Phe Leu
 35 40 45

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

- (ix) FEATURE:
 (A) NAME/KEY: Oligonucleotide probe
 (B) LOCATION: 1-50
 (C) IDENTIFICATION METHOD:
 (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Thr Gly Gly Thr Ala Ala Ala Ala Gly Cys Thr Ala Cys Ala Gly
 1 5 10 15
 Thr Cys Thr Cys Ala Ala Ala Gly Cys Ala Thr Cys Cys Ala Gly
 20 25 30
 Cys Ala Cys Ala Ala Thr Gly Gly Cys Ala Ala Ala Gly Thr Cys
 35 40 45
 Ala Gly Ala Gly Ala
 50

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: hNRG3B1 transmembrane proximal 1
(B) LOCATION: 1-8
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Asn Asp Gly Glu Cys Phe Val Ile
1 5 8

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: hNRG3B1 transmembrane proximal 2
(B) LOCATION: 1-9
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Glu Phe Met Glu Ser Glu Glu Val Tyr
1 5 9

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 466 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: EST Genbank entry H23651
(B) LOCATION: 1-466
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AATTTCTGCC GAAAACTGAT TCCATCTTAT CGGATCCAAC AGACCACTTG 50
GGGATTGAAT TCATGGAGAG TGAAGAACTT TATCAAAGGC AGGTGCTGTC 100
AATTTTCATGT ATCATCTTTG GAATTGTCAT CGTGGGCATG TTCTGTGCAG 150

CATTCTACTT CAAAAGCAAG AAACAAGCTA AACAAATCCA AGAGCAGCTG 200
 AAAGTGCCAC AAAATGGTAA AAGCTACAGT CTCAAAGCAT CCAGCACAAT 250
 GGCAAAGTCA GAGAACTTGG TGAAGAGCCA TGTCCAGCTG CAAAATAAAA 300
 TGTCAGGCTT CTGAGCCCCA GCTAAGCCAT CATATCCCCT GTNGACCTGC 350
 ACGTGCACAT CCNGATGGCC CGTTTCCTGC CTTTTNTGAT GACATTTNCA 400
 CCACAAATGN AGTGAAAATG GGNCTTTTCN TGCCTTAACT GGTGACNTT 450
 TTTNCCCCAA AAGGAG 466

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2091 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: Human NRG3B2 (hNRGB2)
- (B) LOCATION: 1-2091
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGAGTGAAG GGGCGGCCGC TGCCTCGCCA CCTGGTGCCG CTTCGGCAGC 50
 CGCCGCCTCG GCCGAGGAGG GCACCGCGGC GGCTGCGGCG GCGGCAGCGG 100
 CGGGCGGGGG CCCGGACGGC GCGGGCGAAG GGGCGGCCGA GGGGGGGGG 150
 GAGTTACGCT GTAGCGACTG CATCGTGTGG AACCGGCAGC AGACGTGGCT 200
 GTGCGTGGTA CCTCTGTTCA TCGGCTTCAT CGGCCTGGGG CTCAGCCTCA 250
 TGCTTCTCAA ATGGATCGTG GTGGGCTCCG TCAAGGAGTA CGTGCCCACC 300
 GACCTAGTGG ACTCCAAGGG GATGGGCCAG GACCCCTTCT TCCTCTCCAA 350
 GCCCAGCTCT TTCCCCAAGG CCATGGAGAC CACCACCACT ACCACTTCCA 400
 CCACGTCCCC CGCCACCCCC TCCGCCGGGG GTGCCGCCTC CTCCAGGACG 450
 CCCAACCGGA TTAGCACTCG CCTGACCACC ATCACGCGGG CGCCCACTCG 500
 CTTCCCCGGG CACCGGGTGC CCATCCGGGC CAGCCCGCGC TCCACCACAG 550
 CACGGAACAC TGCGGCCCCCT GCGACGGTCC CGTCCACCAC GGCCCCGTTC 600
 TTCAGTAGCA GCACGCTGGG CTCCCGACCC CCGGTGCCAG GAACTCCAAG 650
 TACCCAGGCA ATGCCCTCCT GGCCTACTGC GGCATACGCT ACCTCCTCCT 700

ACCTTCACGA TTCTACTCCC TCCTGGACCC TGTCTCCCTT TCAGGATGCT 750
 GCCTCCTCTT CTCCTCTTC TTCCTCCTCC GCTACCACCA CCACACCAGA 800
 AACTAGCACC AGCCCCAAAT TTCATACGAC GACATATTCC ACAGAGCGAT 850
 CCGAGCACTT CAAACCCTGC CGAGACAAGG ACCTTGCATA CTGTCTCAAT 900
 GATGGCGAGT GCTTTGTGAT CGAAACCCTG ACCGGATCCC ATAAACACTG 950
 TCGGTGCAAA GAAGGCTACC AAGGAGTCCG TTGTGATCAA TTTCTGCCGA 1000
 AAACTGATTC CATCTTATCG GATCCAACAG ACCACTTGGG GATTGAATTC 1050
 ATGGAGAGTG AAGAAGTTTA TCAAAGGCAG GTGCTGTCAA TTTCATGTAT 1100
 CATCTTTGGA ATTGTCATCG TGGGCATGTT CTGTGCAGCA TTCTACTTCA 1150
 AAAGCAAGAA ACAAGCTAAA CAAATCCAAG AGCAGCTGAA AGTGCCACAA 1200
 AATGGTAAAA GCTACAGTCT CAAAGCATCC AGCACAATGG CAAAGTCAGA 1250
 GAACTTGGTG AAGAGCCATG TCCAGCTGCA AAATTATTCA AAGGTGGAAA 1300
 GGCATCCTGT GACTGCATTG GAGAAAATGA TGGAGTCAAG TTTTGTGCGC 1350
 CCCCAGTCAT TCCCTGAGGT CCCTTCTCCT GACAGAGGAA GCCAGTCTGT 1400
 CAAACACCAC AGGAGTCTAT CCTCTTGCTG CAGCCCAGGG CAAAGAAGTG 1450
 GCATGCTCCA TAGGAATGCC TTCAGAAGGA CACCCCGTC ACCCCGAAGT 1500
 AGGCTAGGTG GAATTGTGGG ACCAGCATAT CAGCAACTCG AAGAATCAAG 1550
 GATCCCAGAC CAGGATACGA TACCTTGCCA AGGGTATTCA TCCAGTGGTT 1600
 TAAAAACCCA ACGAAATACA TCAATAAATA TGCAACTGCC TTCAAGAGAG 1650
 ACAAACCCCT ATTTTAATAG CTTGGAGCAA AAGGACCTGG TGGGCTATTC 1700
 ATCCACAAGG GCCAGTTCTG TGCCCATCAT CCCTTCAGTG GGTTTAGAGG 1750
 AAACCTGCCT GCAAATGCCA GGGATTTCTG AAGTCAAAAG CATCAAATGG 1800
 TGCAAAACT CCTATTGAGC TGACGTTGTC AATGTGAGTA TTCCAGTCAG 1850
 CGATTGTCTT ATAGCAGAAC AACAAGAAGT GAAAATATTG CTAGAACTG 1900
 TCCAGGAGCA GATCCGAATT CTGACTGATG CCAGACGGTC AGAAGACTAC 1950
 GAACTGGCCA GCGTAGAAAC CGAGGACAGT GCAAGTGAAA ACACAGCCTT 2000
 TCTCCCCCTG AGTCCCACAG CCAAATCAGA ACGAGAGGCG CAATTTGTCT 2050
 TAAGAAATGA AATACAAAGA GACTCTGCAT TGACCAAGTG A 2091

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 696 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(ix) FEATURE:

- (A) NAME/KEY: Human NRG3B2
- (B) LOCATION: 1-696
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met	Ser	Glu	Gly	Ala	Ala	Ala	Ala	Ser	Pro	Pro	Gly	Ala	Ala	Ser	
1				5				10						15	
Ala	Ala	Ala	Ala	Ser	Ala	Glu	Glu	Gly	Thr	Ala	Ala	Ala	Ala	Ala	
				20				25						30	
Ala	Ala	Ala	Ala	Gly	Gly	Gly	Pro	Asp	Gly	Gly	Gly	Glu	Gly	Ala	
				35				40						45	
Ala	Glu	Pro	Pro	Arg	Glu	Leu	Arg	Cys	Ser	Asp	Cys	Ile	Val	Trp	
				50				55						60	
Asn	Arg	Gln	Gln	Thr	Trp	Leu	Cys	Val	Val	Pro	Leu	Phe	Ile	Gly	
				65				70						75	
Phe	Ile	Gly	Leu	Gly	Leu	Ser	Leu	Met	Leu	Leu	Lys	Trp	Ile	Val	
				80				85						90	
Val	Gly	Ser	Val	Lys	Glu	Tyr	Val	Pro	Thr	Asp	Leu	Val	Asp	Ser	
				95				100						105	
Lys	Gly	Met	Gly	Gln	Asp	Pro	Phe	Phe	Leu	Ser	Lys	Pro	Ser	Ser	
				110				115						120	
Phe	Pro	Lys	Ala	Met	Glu	Thr	Thr	Thr	Thr	Thr	Thr	Ser	Thr	Thr	
				125				130						135	
Ser	Pro	Ala	Thr	Pro	Ser	Ala	Gly	Gly	Ala	Ala	Ser	Ser	Arg	Thr	
				140				145						150	
Pro	Asn	Arg	Ile	Ser	Thr	Arg	Leu	Thr	Thr	Ile	Thr	Arg	Ala	Pro	
				155				160						165	
Thr	Arg	Phe	Pro	Gly	His	Arg	Val	Pro	Ile	Arg	Ala	Ser	Pro	Arg	
				170				175						180	
Ser	Thr	Thr	Ala	Arg	Asn	Thr	Ala	Ala	Pro	Ala	Thr	Val	Pro	Ser	
				185				190						195	
Thr	Thr	Ala	Pro	Phe	Phe	Ser	Ser	Ser	Thr	Leu	Gly	Ser	Arg	Pro	
				200				205						210	

Pro	Val	Pro	Gly	Thr 215	Pro	Ser	Thr	Gln	Ala 220	Met	Pro	Ser	Trp	Pro 225
Thr	Ala	Ala	Tyr	Ala 230	Thr	Ser	Ser	Tyr	Leu 235	His	Asp	Ser	Thr	Pro 240
Ser	Trp	Thr	Leu	Ser 245	Pro	Phe	Gln	Asp	Ala 250	Ala	Ser	Ser	Ser	Ser 255
Ser	Ser	Ser	Ser	Ser 260	Ala	Thr	Thr	Thr	Thr 265	Pro	Glu	Thr	Ser	Thr 270
Ser	Pro	Lys	Phe	His 275	Thr	Thr	Thr	Tyr	Ser 280	Thr	Glu	Arg	Ser	Glu 285
His	Phe	Lys	Pro	Cys 290	Arg	Asp	Lys	Asp	Leu 295	Ala	Tyr	Cys	Leu	Asn 300
Asp	Gly	Glu	Cys	Phe 305	Val	Ile	Glu	Thr	Leu 310	Thr	Gly	Ser	His	Lys 315
His	Cys	Arg	Cys	Lys 320	Glu	Gly	Tyr	Gln	Gly 325	Val	Arg	Cys	Asp	Gln 330
Phe	Leu	Pro	Lys	Thr 335	Asp	Ser	Ile	Leu	Ser 340	Asp	Pro	Thr	Asp	His 345
Leu	Gly	Ile	Glu	Phe 350	Met	Glu	Ser	Glu	Glu 355	Val	Tyr	Gln	Arg	Gln 360
Val	Leu	Ser	Ile	Ser 365	Cys	Ile	Ile	Phe	Gly 370	Ile	Val	Ile	Val	Gly 375
Met	Phe	Cys	Ala	Ala 380	Phe	Tyr	Phe	Lys	Ser 385	Lys	Lys	Gln	Ala	Lys 390
Gln	Ile	Gln	Glu	Gln 395	Leu	Lys	Val	Pro	Gln 400	Asn	Gly	Lys	Ser	Tyr 405
Ser	Leu	Lys	Ala	Ser 410	Ser	Thr	Met	Ala	Lys 415	Ser	Glu	Asn	Leu	Val 420
Lys	Ser	His	Val	Gln 425	Leu	Gln	Asn	Tyr	Ser 430	Lys	Val	Glu	Arg	His 435
Pro	Val	Thr	Ala	Leu 440	Glu	Lys	Met	Met	Glu 445	Ser	Ser	Phe	Val	Gly 450
Pro	Gln	Ser	Phe	Pro 455	Glu	Val	Pro	Ser	Pro 460	Asp	Arg	Gly	Ser	Gln 465
Ser	Val	Lys	His	His 470	Arg	Ser	Leu	Ser	Ser 475	Cys	Cys	Ser	Pro	Gly 480
Gln	Arg	Ser	Gly	Met 485	Leu	His	Arg	Asn	Ala 490	Phe	Arg	Arg	Thr	Pro 495

Pro	Ser	Pro	Arg	Ser	Arg	Leu	Gly	Gly	Ile	Val	Gly	Pro	Ala	Tyr	
				500					505					510	
Gln	Gln	Leu	Glu	Glu	Ser	Arg	Ile	Pro	Asp	Gln	Asp	Thr	Ile	Pro	
				515					520					525	
Cys	Gln	Gly	Tyr	Ser	Ser	Ser	Gly	Leu	Lys	Thr	Gln	Arg	Asn	Thr	
				530					535					540	
Ser	Ile	Asn	Met	Gln	Leu	Pro	Ser	Arg	Glu	Thr	Asn	Pro	Tyr	Phe	
				545					550					555	
Asn	Ser	Leu	Glu	Gln	Lys	Asp	Leu	Val	Gly	Tyr	Ser	Ser	Thr	Arg	
				560					565					570	
Ala	Ser	Ser	Val	Pro	Ile	Ile	Pro	Ser	Val	Gly	Leu	Glu	Glu	Thr	
				575					580					585	
Cys	Leu	Gln	Met	Pro	Gly	Ile	Ser	Glu	Val	Lys	Ser	Ile	Lys	Trp	
				590					595					600	
Cys	Lys	Asn	Ser	Tyr	Ser	Ala	Asp	Val	Val	Asn	Val	Ser	Ile	Pro	
				605					610					615	
Val	Ser	Asp	Cys	Leu	Ile	Ala	Glu	Gln	Gln	Glu	Val	Lys	Ile	Leu	
				620					625					630	
Leu	Glu	Thr	Val	Gln	Glu	Gln	Ile	Arg	Ile	Leu	Thr	Asp	Ala	Arg	
				635					640					645	
Arg	Ser	Glu	Asp	Tyr	Glu	Leu	Ala	Ser	Val	Glu	Thr	Glu	Asp	Ser	
				650					655					660	
Ala	Ser	Glu	Asn	Thr	Ala	Phe	Leu	Pro	Leu	Ser	Pro	Thr	Ala	Lys	
				665					670					675	
Ser	Glu	Arg	Glu	Ala	Gln	Phe	Val	Leu	Arg	Asn	Glu	Ile	Gln	Arg	
				680					685					690	
Asp	Ser	Ala	Leu	Thr	Lys										
				695	696										